Effects of Beta Cyclodextrin and Pluronic F-127 on the stability of model proteins: Lysozyme, Insulin and Antibodies

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ABSTRACT

The goal of protein therapeutic formulation research is to create effective solutions in which the protein activity is maintained in solution and is stable throughout the shelf life.

Stabilisation of protein in dry and solution forms under certain conditions like temperature, pH has been a challenge in protein therapeutic drug production.

Investigating therapeutic protein physiochemical properties and aggregation are critical for development of formulations for enhancement of the drug efficacy and safety. Challenges in the analysis are usually compounded in the formulation with various excipients, small volumes and high concentrations.

The aim of this research work is to show the effects of beta cyclodextrin and pluronic F-127 on the stability of protein on lysozyme, insulin and antibody (Immunoglobulin), as well as their effects of concentration, and other solution conditions. The drying (spray-dried (SD); electrosprayed (ESD)) effect on lysozyme formulations with or without excipients was also investigated. High sensitivity differential scanning calorimetry (HSDSC), Differential scanning calorimetry (DSC) and other characterization techniques such as Dynamic light scattering (DLS), Thermogravimetry analysis (TGA), High performance liquid chromatography (HPLC), Scanning electron microscope (SEM) and Ultraviolet-Visible (UV-Vis) spectrophotometry were applied to assess the thermal stability and aggregation of these proteins including and excluding excipients. Beta cyclodextrin and pluronic F-127 demonstrated several promising qualities on lysozyme with the drying technique applied. Addition of beta cyclodextrin to spray dried lysozyme improved and maintained the biological activity.

Furthermore, the presence of beta cyclodextrin and pluronic F-127 maintained the denaturation temperature of spray dried lysozyme. A combination of both excipients with the drying technique exhibited a form of stability by maintaining the enzymatic activity of lysozyme. Electrospray technique in the presence and absence of excipients improved lysozyme stability.

The thermogram obtained for Insulin in phosphate buffered saline thermal denaturation was shifted to an increased Tm temperature of 81.2°C in the presence of pluronic F-127 at 10°C scan rate compared to unprocessed insulin at 78.44°C. In the same instance, beta cyclodextrin was not effective on the thermal stability of the insulin, rather, a reduced effect at Tm of 74.06°C and 71.45°C respectively was observed at both scan rates (10°C and 60°C). A change in the conformation of insulin affected the transition peak's shape, sharpness, and position. Pluronic F-127 was able to enhance or preserve the helical structure of insulin by producing a stabilizing effect. No aggregation was observed for 1:1%w/v insulin, pluronic F-127 dissolved in phosphate buffered saline. Beta cyclodextrin and pluronic F-127 combination could cause the increase in the activity and stability of lysozyme using the correct formulation and technique following results obtained from electrosprayed technique. The thermogram of the antibody (IgG) showed a transition followed by a sharp aggregation peak. The thermograms showed different reactions to pH, as the effect of pH on thermal stability was investigated at pH 5.5 and 7.4 from the aggregation investigation. Increase in pH (7.4) did not show a difference in the denaturation temperature when compared to pH 5.5. Both excipients appeared to improved protein stability in their unique ways.

Keywords: Lysozyme, insulin, monoclonal antibody, protein formulations, excipients, stability, aggregation, high sensitivity differential scanning calorimetry, differential scanning calorimetry, dynamic light scattering, scanning electron microscope, size exclusion high performance liquid chromatography.

LIST OF ABBREVIATIONS

DSC - Differential scanning calorimetry

HSDSC- High sensitivity differential scanning calorimetry

TGA- Thermogravimetry analysis

SEM - Scanning electron microscope

UV-Vis Ultra violet- visible spectroscopy

SEC - Size exclusion chromatography

ESD -Electrospray

DLS- Dynamic light scattering

SD - Spray dry

RNase A- Ribonuclease A

IgG- Immunoglobulin

ICH- International conference of harmonisation

FDA- Food and drug administration

pH- potential of hydrogen

ΔCp- change in heat capacity

β cyclodextrin- Betacyclodextrin

ΔS – change in Entropy

ΔH- change in Enthalpy

ΔG- change in free energy

Zn-Zinc

Cd-Cadmium

Pb-Lead

Tm- thermal denaturation temperature

KV- Kilo Volt

mA- milliampere

IU- International unit

WHO- World health organisation

MW-Molecular weight

PI -Isoelectric point

kDa -Kilo Dalton

Tg- glass transition

CE- Cation exchange

CE-SDS- Capillary electrophoresis sodium dodecyl sulfate (CE-SDS)

HPLC- High-performance liquid chromatography

LOD- limit of detection

SD- Standard deviation

TFA- Trifluoroacetic acid

PPO- Poly (propylene oxide)

PEO -Polyethylene oxide

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CONFERENCES ATTENDED DURING THE RESEARCH.

Conference: MIBio 2019, Downing college Cambridge

Focus: Stability of Biopharmaceutical from molecular interaction to successful product.

Date: 13th November 2019

Conference: PharmSci360, Walter Washington DC

Organizer: American association of Pharmaceutical Science

Focus: Advancing Pharmaceutical science career and Opportunity

Date: 4th -7th November 2018

Conference: Postgraduate Research Conference. University of Sunderland

Organizer: Postgraduate research development

Focus: Writing for publication delivered by Rob Patterson from sage publications

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Conference: International Pharmaceutical Technology Conference 2017, London UK

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Conference: Teaching and Learning Conference. University of Sunderland

Organisers: Academic development in collaboration with academic community

Focus: Sharing your work and celebrating teaching

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Conference: Regional Pharmacy conference

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- Thermal stability of insulin**.** abstract publication and poster presentation at the MIBio 2019 conference, Downing college Cambridge

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1.0 GENERAL INTRODUCTION

1.1 Protein Therapeutics

Protein-based therapeutics are presently highly sought after for their pharmacological effect and its significant role in treatment of diseases in the world of medicine (AlQahtani et al., 2019). Hundreds of therapeutic protein medications are intended for use in clinicals in the European Union, United States of America including other parts of the world (Dimitrov, 2012). The therapeutic proteins presently in the market are predominantly recombinant proteins and they are mostly applied for immune disorders, inflammation, infections, cancer therapy (Zhang et al., 2020) and metabolic disorders (Roberts, 2014). Examples includes monoclonal antibody (Secher et al., 2022), insulin, lysozyme and others. Therapeutic proteins are either produced naturally by the body or engineered in a laboratory and administered to patients with the goal of improving or treating a disease, they are often obtained from microbial cells or genetically modified animals or plants (Kontermann, 2010). Based on their pharmacological effects, therapeutic proteins are classified in terms of their clinical uses. They are used in the replacement of abnormal and deficient proteins (Chakraborty et al., 2021; Leader et al., 2008), as protein deliveries and existing pathway augmentation (Dimitrov, 2012). In addition, therapeutic proteins are grouped based on molecular types such as enzymes, hormones, blood and growth factors, interferons, engineered protein scaffolds, thrombolites, bone morphogenetic proteins, interleukins Fc fusion proteins and antibody-based drugs (Carter, 2011). The native structure of therapeutic proteins is required for biological activities, including the conformation, which is particularly crucial in the development of protein drugs and organic compounds made up of α-amino [alpha-amino] carboxylic acid (also known as amino acids) plays in the body, a critical part.

Human or regular insulin was genetically the first engineered crossover therapeutic protein manufactured to treat diabetes mellitus in 1978 (Rosenfeld, 2002; Falconer, 2019), and was

approved in 1982. In addition, in 1985, muramic IgG2a isotype, a monoclonal antibody therapeutic, was the first of its kind, approved for the treatment of T-cell-mediated rejected renal allografts (Campuzano and Sandoval, 2021), however, was later withdrawn from the market. To date, there has been an approval of a total of 239 peptide and therapeutic protein drugs by the FDA, in United States for clinical use and many other promising therapeutics proteins are still on clinical trials (Chakraborty et al., 2021). Proteins like monoclonal antibodies are categorised based on their molecular method of action, which includes non-covalently binding to targets (Dimitrov, 2011). Protein size is a vital physical characteristic as particle size analyser are used in the study of protein to discuss and interpret molecular weight and protein size. They are classified as sensitive biological molecules employed in a wide range of therapeutic and diagnostic medicines as well as biological commodities productions. The high sensitivity of protein is largely owing to intricacies of its manufacturing, which includes restricted chemical and purification processes, making it very unstable as a result of precipitation and denaturation. Protein aggregation may reduce protein functionality (Lu et al., 2022).

1.1.1 Proteins

Proteins are macromolecules also known as polypeptides, heterogeneous in their native environment that is extremely important in biological systems (Chetachukwu et al., 2022). Sources of protein include meat, milk, legume, egg cereal. The majority of the work is done in cells, the body's tissue and organs are necessary for regulations, function and structure. Several small units of protein are arranged into globular forms and arranged in a linear pattern. Twenty different natural acids are combined to produce amino acid sequence and are therefore required for human growth and metabolism. Amino acids are also important for the maintenance and repair of tissue. The absence of any of these amino acids might impair the

ability of the tissue to grow, heal, or sustain itself (Hoffman and Falvo, 2004). They further serve as major structural components of the tissues and other parts of the body. Proteins must be metabolized and converted to amino acid for its use in the body (Hoffman and Falvo, 2004). The various forms of protein act as contractile protein, defensive, enzymatic, hormonal, storage, structural, transport and receptor, these are all present in every tissue and cell with specialised functions for standard development (Jamerson, 2018).

1.1.2 Protein Structure

Protein is a primary structure formed with various chains of amino acids. Protein has a unique 3-dimentional structure with specified functions (Jamerson, 2018). It is made up of amino acid polymers, which are chains or smaller units of amino acids (George, 1992). They also have peptide backbones, or primary chains of repeating units, with varying side chain attachments connected with peptide bond. Every one of the proteins has a distinct side chain concatenation that dictates the properties of the specific chain (George, 1992). The amino acids sequences illustrated in (Figure 1.1) include the protein's basic primary structure, secondary structure, also known as the alpha helix, and Beta-sheet structure; Secondary structures are three-dimensional elements with a protein backbone orientation, followed by tertiary structures formed from secondary structural elements (Cleland and Craik, 1996), and finally quaternary structures made up of several subunits with tertiary structures (Cleland and Craik, 1996; Saito and Kobayashi, 1999. Proteins are made up of one or more polypeptide molecules, some proteins are normally inactive in their natural state, but they require the assistance of secondary, tertiary, and quaternary structures to execute certain functions (Zuchner, 2012). The native form of protein, based on how it is configured, is followed by the allocation of the alpha helix and beta-sheet, which results in its secondary conformation (Elkordy et al., 2008) and sometimes connected by hydrogen bond. Interactions which take place non-covalently (Ata-scientific 2020) such as hydrophobic packing, hydrogen bonding, ionic strength, and

Vander Waals forces cause proteins to fold into one or more specific conformations for biological functions to be performed (Elkordy et al., 2008). The native form determines the configuration, after which is the allocation of the alpha helix and beta-sheet, which results to secondary conformation (Elkordy et al., 2008) and connected sometimes by hydrogen bond.

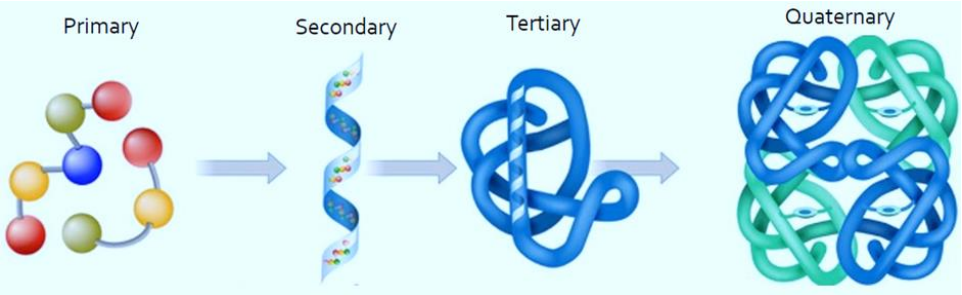


Figure 1.1: Illustration showing structures of protein, https://www.dreamstime.com/illustration/protein.html

1.2 Formulation and development of therapeutic proteins

A formulation stage requires a buffering agent to manage pH and other excipients, for therapeutic protein concentration and solution to be achieved, example of such excipients include amino acid, salts, polyols and surfactants (Pfister et al., 2018; Zhang et al., 2020). This process takes place in the development phase in drug process development (Figure 1.2).

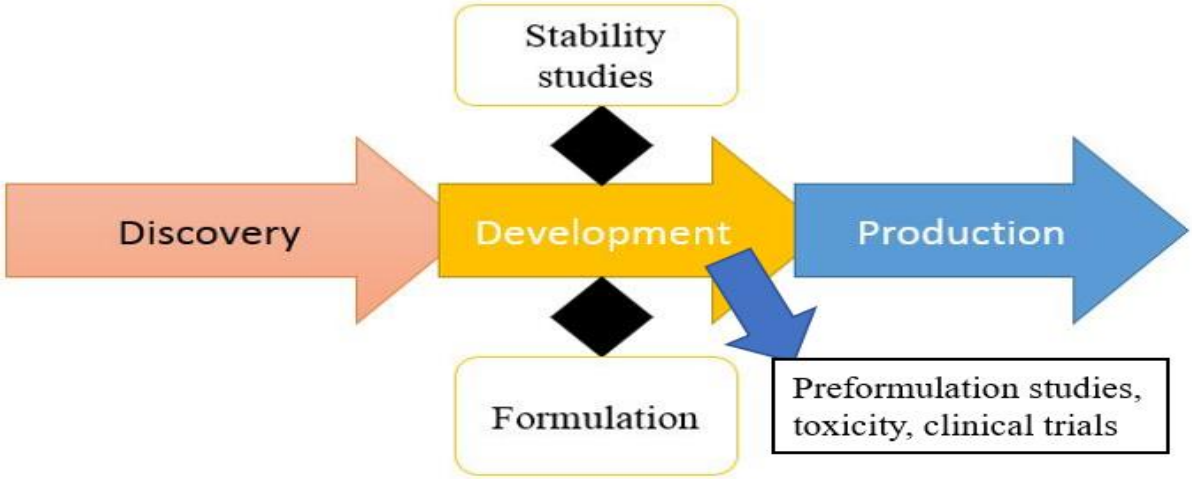


Figure 1.2: Illustration of the process of drug development with focus on the formulation and stability at the development stage.

Biopharmaceutical products need a sufficient shelf life, which can only be achieved with a properly formulated solution as aggregate formation may impact negatively on the efficacy of a medicine and the medication safety (Pfister et al., 2018). There are several methods of purification such as gel-filtration chromatography, ion-exchange chromatography and techniques for analysis that are required for the total characterization of protein therapeutic, its higher order structure, and any other related impurities as a biotherapeutic drug progresses from research to development phase and then to clinic. Circular dichroism, differential scanning calorimetry, differential scanning fluorimetry, dynamic light scattering, fourier transform infrared raman spectroscopy, gel- and capillary electrophoresis, hydrophobic interaction chromatography, ion exchange chromatography, and size exclusion chromatography are examples of such analytical techniques (Campuzano and Sandoval, 2021. A complete understanding of a protein's basic characteristics, such as pI, protein purity, stabilisers and solubility, is required for successful protein formulation development. The purity of a protein should be appropriate before doing any pre-formulation tests; typically, with a level of purity of 95% or more, advised. (Berry, 1996).

Formulation development of proteins are faced with various challenges, one of which includes low solubility which in most cases are combined with strong adsorption tendencies (Hawe and Freb, 2007). The pioneers in insulin development also faced challenges in producing a sufficient unadulterated product, with adequate shelf life, in a suitable formulation as injections for patients. Protein such as insulin is instead converted to a dormant but stable form using zinc ions, enabling the development of formulations with different release rate of the active ingredient (Falconer, 2019; Teska et al., 2014). Pharmaceutical protein formulations are designed in various types of dosage form for parenteral mode of delivery since the bioavailability of these drugs are poor when administered through certain route like oral delivery (Teska et al., 2014; Zhu et al., 2021). A variety of diseases like different forms of

cancer and rheumatism are currently being treated with parenteral therapeutic proteins (Falconer, 2019). Researchers have put in tremendous effort to improve protein formulations, leading to the development of effective formulas (Lee, 2002). Safety and efficacy are requirements to be met for protein delivery technology success. Producing an oral dosage form is a convenient route for drug administration and improves the absorption of drugs making it the most common formulation however, it is faced with challenges during production. Restriction faced in oral formulation of protein development are impermeable mucosal tissue present in the intestine and the metabolic enzyme. (Lee, 2002), drug solubility, and stability in the gastrointestinal tract environment (AlQuahtani et al., 2021). As a result, they are produced in liquid (solution) and solid (powdered) forms for reconstitution. Most proteins are generally unstable in solutions and are easily affected by stress during manufacturing, processing, and storage. Even though the liquid formulations are commonly used for protein therapeutic formulations, the solid formulations are more stable (Falconer et al., 2011). Currently in the industry, it is observed that liquid formulation of protein is cheaper and easily used compared to formulations that are lyophilized. (Falconer et al., 2011; AlQuahtani et al., 2021). Although liquid formulations are favoured for injectable medicines due to their ease of preparation in both production and for end users, (Siew, 2014) however, subjecting proteins to aggregation and denaturation under certain stress conditions, had shown liquid formulations for injectable medicines not practical. Due to the instability, proteins may be produced in solid forms and administered as a medication via injection rather than oral delivery, protein in solid form are made through drying methods (Forbes et al., 2007) such as spray drying, electrospray, crystallization and freeze drying.

1.3 Stability study

Protein stability is one of the most limiting criteria in the development and testing of biopharmaceuticals, as it requires special attention during the preformulation phase and product development process (Butreddy et al., 2021). Before submitting a registration dossier, it is now mandatory to conduct stability studies on new drug moiety. Among the stability studies conducted are accelerated (6-month) and long-term (12-month) stability studies. However, it is possible to conduct intermediate studies, which last for six months, under less rigorous conditions than accelerated studies.

Stability studies are frequently carried out under accelerated stressful circumstances to speed up formulation development (Wang, 1999), such conditions include high humidity and temperature and extreme pH. The studies are usually brief and intensive, but they are quite useful in the screening of protein formulations (Wang, 1999).

The ICH and FDA detail guidelines and requirements for stability testing data to enable understanding of how drug product and drug substance quality change over time as a result of various environmental conditions (Blessy at al., 2014).

Stability and forced degradation studies are carried out during drug development process and used in the generation of formulations for stability- indication assay methods (Sonawane et al., 2016), the stability knowledge of the drug molecules helps in the selection of proper package, storage and formulation and stress testing. According to ICH recommendations, it is used to identify potential degradation products, which aids in assessing the inherent stability of the molecule and developing degradation routes, as well as validating the stability indicating methodologies used (ICH guidelines, Q1A (R2) (2003). Long term tests samples stored under extreme conditions like 80°C (Banker and Rhodes, 2002) at different periods of time are

analysed at regular intervals. (Wang, 1999). Changes in the state of the products are easily recognised as there are changes or shifts in the outcome of the results during measurement**.**

The food and drug agency (FDA) recommendation for stability testing is that all assay procedures for stability are stability-indicating. The stability indicating methods ensure the safety, efficacy and quality of the product and monitors result during stability (Blessy at al., 2014). They are analytical methods that are quantitatively validated to detect a change with time in properties of the drug and ensure that the degradation products and the active ingredients are measured accurately without interfering (Sonawane et al., 2016). Stability indicating methods analyse formulations using analytically developed technologies that are constantly being manufactured such as a variety of chromatography techniques, like capillary electrophoresis chromatography (CEC), gas chromatography (GC), reversed phase high performance liquid chromatography (RP-HPLC), super critical fluid chromatography (SFC), and thin-layer chromatography (TLC), can be used to separate and analyse the unknown impurity found in drug products (ICH Guidance for Industry, Q6B, 1999).

Decomposition reactions are investigated kinetically by carrying out sample measurements with different heating rates. The results give certain expectations about the decomposition behaviour within a short period of time. The following parameters such as activity of aggregation, colour, protein degradation rate, precipitation, pH and viscosity are used in monitoring stability studies (Wang, 1999). High temperatures are frequently used in accelerated stability experiments. Differential scanning calorimetry and thermogravimetry analysis are examples of techniques approved for stability studies.

1.3.1 Stability and Instability of Therapeutic Protein

The stability of proteins and peptides is a very important consideration in formulation development as proteins are extremely vulnerable to the effects of chemical (Manning et al.,

1989, Goolcharran et al., 2000, Brange et al, 2000) and physical degradation (Jacob et al., 2006; Butreddy et al., 2021). The best stability comes from choosing the right buffer, excipients, pH, and temperature. Stability in protein is the resultant effect of an equilibrium between stabilizing and destabilizing agents. The stabilizing agents and destabilizing agents are caused by protein-solvent/intra-protein interaction and significant increase in entropy of unfolding respectively (Challener, 2015). In this regard, it is essential to have a knowledge of the origins of protein stability whilst understanding their function and structure. The stability must be great enough for the protein to find and maintain its native conformation relative to other conformations but not so great that conformational changes or adjustments, considered an integral part of many protein functions are disallowed (Schellman and Becktel, 1987).

The stability of a native protein is determined at conditions where the protein is folded and active. Accuracy in the measurement of protein stability is important in understanding the underlying factors and interactions that stabilize a protein structure and how protein is manipulated to be more (or less) stable (Hamborg et al., 2020). Spectroscopic or calorimetric techniques are used for stability of protein analysis via taking a measurement of the unfolding and a gradual adjustment of the temperature or concentration of a chemical denaturant. Some proteins require elevated temperature to completely unfold in a chemical denaturant (Hamborg et al., 2020), Proteins encounter degradation during biopharmaceutical production process thereby leading to protein denaturation causing structural changes and loss of activity of the molecules making them unstable. Protein instability is affected by various exposures to atmospheric conditions like elevated temperature, heat, pressure, surface adsorption (Ohtake et al., 2011), and low pH (Pfister et al., 2018). The structure of protein is so complex physically as well as chemically for maintaining the protein stability for a long time. Hence, the native structure and domains must adapt to changes in certain factors like temperature, pH and ionic strength. The instability of protein occurs usually during production, purification, transportation and storage. It is important to note that water also may influence structural

stability (Thakkar et al., 2012) and mediate deamination in protein formulations. Conformational stability, colloidal stability and chemical stability are the three different kinds of stability. The ability of a protein to retain its natural structure while also folding in the correct manner is referred to as its conformational stability. This can be affected by the physical pathway known as aggregation, precipitation, denaturation, and adsorption at interfaces. Chemical stability is the stability among amino acids, covalent bonds and different protein domains affected by oxidation (Elkordy, 2002), beta-elimination, deamidation, racemization, disulfide formation and peptide bond hydrolysis, while the tendency of a protein's native structure to resist precipitation, aggregation, and phase separation is known as colloidal stability (Hui et al., 2014).

1.3.1.1 Challenges in Protein Stability

A major challenge with biopharmaceuticals is achieving protein stability. The pharmaceutical industry spends millions of pounds on research trying to find a lasting approach to drug stability as they are faced with problems involving manufacturing, processing and storage of protein. For a stable and effective formulation, it is essential to take into account the various protein instability routes. (Clarkson et al., 2016) Peptide covalent bonds can be formed or destroyed in the course of chemical instability, resulting in the development of new kinds of chemicals (Jacob et al., 2006). In most instances, deamidation, oxidation, and isomerization are the most common types of chemical degradation. However, research that has been carried out over the years suggests that chemical degradation can occur in a number of different ways (Strohl and Strohl, 2012). Asparagine and glutamine, both of which are uncharged polar amino acids, can be deamidated to form glutamic acid, which can then be converted into either aspartate or isoaspartate (Sydow et al., 2014; Daugherty and Mrsny, 2006). In addition to a protein's main sequence and three-dimensional structure, according to Robinson and Robinson, (2001), several solution parameters such as ionic strength, temperature, buffer ions and pH have effects on deamidation (Robinson and Robinson, 2001). Deamidation was also

explained by Daugherty and Mrsny (2006) as a process that normally happens at neutral and basic pHs, with basic conditions accounting for three times as much deamidation as acidic conditions (Daugherty and Mrsny, 2006). Figure 1.3 shows a schematic representation of challenges encountered with protein stability. The chemical change, known as isomerization, has a negative impact on attachment for the target due to amino acid isomerization, which occurs most frequently with aspartic acid. This isomerization has a negative impact on affinity for the target (Dick et al., 2010). If the protein's main solvent dielectric strength, sequence, excipient and additional buffered agents mixes are strong enough to increase isomerization, then neutral or acidic conditions are the most common circumstances under which isomerization occurs. This is because isomerization occurs at a higher rate in neutral conditions than in acidic ones (Sreedhara et al., 2012; Dick et al., 2010).

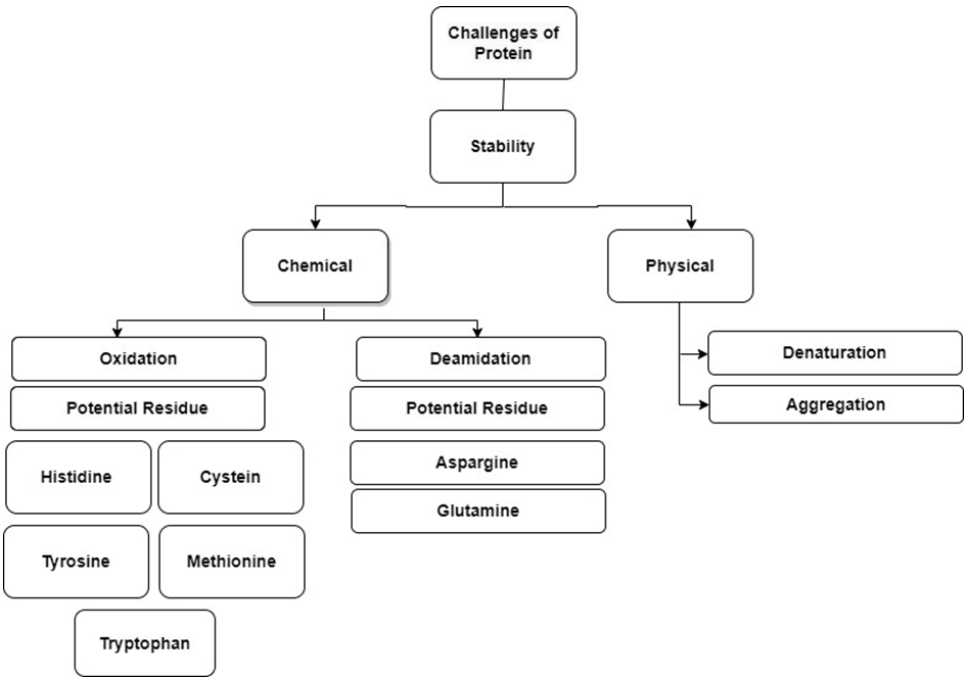


Figure 1.3 Schematic representation of protein challenges. Jain et al., 2019

1.3.2. Folding reversibility and unfolding of protein.

The history of protein unfolding began when Anfinsen and co-workers described the folding of ribonuclease A (RNase A), It was ascertained that RNase A could fold without help from other biological machinery. It was further revealed that spontaneous folding of RNase A proceeds downhill to the lowest free energy polypeptide conformation (Anfinsen, 1973). The native structure of a protein, according to Anfinsen, is the thermodynamically stable structure; the amino acid sequence and solution conditions are the only factors affecting it, not the kinetic folding process. There is little evidence that the native structure of a protein is influenced by whether the protein was created biologically, with the aid of chaperone molecules, or merely refolded in a test tube. Although there are a few exceptions, such as serpins (Wang at al., 1996) insulin, and α-lytic protease (Sohl et al., 1998), serpins are kinetically bound to the biologically active form (Dill et al., 2008).

A protein's native conformation is a unique three-dimensional structure that folds in formulations (Mukherjee and Anand, 2013). Although the chemical characteristics of their amino acids let most proteins fold on their own, some require molecular companions. (Hawe and Freb, 2007). Inclusion bodies containing the protein of interest in an inactive aggregated state are common in bacteria with high protein expression levels. An extra step in the manufacturing process is required to adequately solubilize and fold the therapeutic protein. In order to refold proteins, the buffer used to solubilize them is changed to one that favours the protein's natural state. Chromatographic columns have been found to speed up this process, but the underlying mechanisms of matrix-assisted refolding have not yet been fully understood (Pfister et al., 2018). During the preparation of proteins as pharmaceutical products, considerations are very necessary on the stability of the native conformation of protein. As the stability involves the three-dimensional state and for biological activity to occur, the three-dimensional, folded, and tertiary states must all be stable. Proteins must have a folding mechanism or a state that they may return to within a short time from being an unfolded,

denatured conformation, in order to be stable. Polar groups are more exposed to the solvent while non-polar groups are less exposed during folding (Elkordy et al., 2002).
Although this is not always the case, protein unfolding typically occurs during the transition from its native state to its denatured state (Kocherbitov and Arnebrant, 2006), there is a state of equilibrium between the folded and unfolded conformations of molecules when they are in an aqueous solution.

Determining the stability of the native conformation of protein requires taking into account the thermodynamic relationship between ΔS and ΔH, as well as the magnitude of the system's Gibbs free energy. If the value of ΔG is smaller than zero (negative), this suggests that the native conformation is more stable than the denatured state; consequently, greater stability could produce a negative value of ΔG. Forces that enable stability of protein are broken during the unfolding process, and at higher temperatures when entropy is the most important factor, conformational entropy will win out over any stabilising forces that are present. The unfolding energy (ΔH) that is caused by heat denaturation is what the DSC measures (Gill et al., 2010; Dragan, 2007). Details of protein unfolding can be found in Englander et al., 2007.

Many forces involved in protein folding include the intrinsic propensity of Van der Waals forces, electrostatic interaction (charge pairing and ion repulsion), hydrogen bonding (a strong dipole-dipole attraction, non-covalent in nature bonded to hydrogen atoms and other strongly electronegative atom like nitrogen and oxygen) hydrophobic interaction which is the repulsive interaction between non- polar residues in protein and water, leading to minimal hydration of the hydrophobic core (Dill, 1990). As charged residues are localised in high-dielectric areas on the protein surface, electrostatic interactions between charged side chains are unlikely to play a major factor in protein folding. When it comes to protein stability, pH (near neutral) and salt content tend to have little effect on the structure and stability of the protein (Dill et al.,

2008). Hydrogen-bonding interactions are crucial because in most cases, all conceivable hydrogen-bonding interactions are met in the native structure. Secondary structure such as amide and carbonyl groups on the backbone have been shown to have hydrogen bonds as strong as 1–4 kcal/mol (Byrne et al., 1995) in investigations of mutations in various solvents (Auton et al., 2007). Van der Waals interactions are also crucial because of the tight packing of proteins (Chen and Stities 2001). There are heavily connected interactions with increased capacity of heat (Dill, 1990; Wang, 1999; Rodler et al., 2019).

1.3.3 Protein Denaturation

Denaturation can occur when secondary or tertiary structure is disturbed and the previously buried amino acid are exposed, making it more reactive leading to the loss of the amino acid's native or original properties (Joseph, 2000). It causes protein to become insoluble in solvents where it was previously soluble. If a denaturation reaction occurs, the denatured protein remains in solution after being removed from the isoelectric point. Furthermore, if denaturation occurs near or at an isoelectric point, the denatured protein directly clusters (Joseph, 2000). Coagulation and denaturation are sometimes interchangeably applied, and coagulation is at times incorrectly used as agglutination, flocculation, and precipitation. However, in this research study, an emphasis will be placed on precipitation and denaturation because these processes are important during protein stability. Protein can be said to have precipitated when it is separated from a solution in which it was suspended without undergoing any changes. (Eisenberg and Richard, 1995). Further, the number of chemical changes in protein molecule, make each of the precipitation methods reversible processes (Eisenberg and Richard,1995). The precipitate agent is either diluted or eliminated before the process continues, though precipitates are dissolved instantly. However, when insoluble compounds precipitate, they frequently go through additional changes that render them irreversible if they

are left in the precipitating agent for an exceptionally long time, because the precipitating agent is a solvent (Eisenberg and Richard, 1995). Proteins are most certainly re-dissolved after the removal the precipitated agent. The DSC is utilized for the measurement of protein denaturation which measures the excess heat capacity of unfolding and gives a direct measurement of the enthalpy for folding as well as the melting temperature (Alexander and Hughes, 1995, Hughes and Richberg, 1993). DSC is only able to provide a comprehensive thermodynamic detail of the process of folding if the process is reversible. This is accomplished by fitting DSC experiments and determination of the stability thermodynamically at room temperature (Hamborg et al., 2020).

1.3.3.1 Causes of Denaturation

Denaturation occurs when a protein's folded structure is disturbed and impacted by interactions on the surface, changes in chemical compositions as well as changes in thermal properties and pH (Cleland et al., 1993; Butreddy et al., 2021). The loss of protein structure and/or denaturation could be caused by aggregation via hydrophobic interactions (Cleland et al., 1993), so protein structure longevity is majorly and most effectively maintained and preserved when it is in its solid form. However, there are still significant challenges affecting protein formulations as dehydration is still able to cause the structure of protein to be rearranged and unfolding when in lyophilised forms. Disulphide bonds that are broken by reducing agents are contained in antioxidants which should not be added to protein formulations on their own. Many surfactants, like Tween 20 and Tween 80 and poloxamer, have residual peroxides that can oxidise amino acids.

1.3.4 Protein Aggregation

Protein aggregation is another challenge faced when preparing protein therapeutics (Alex and Daniel, 2011). Protein-based medications are often manufactured at high protein

concentrations, which encourages aggregates to develop, making long-term stability of the final product difficult to guarantee (Pfister et al., 2018). They occur both in liquid and solid state leading to reduced activity (Roberts, 2014). Aggregates are a major risk factor for the unwanted immunogenicity of therapeutic antibodies, which can lead to immune-mediated side effects (Secher et al., 2022). Protein molecules gather to form complexes that are stable and made up of two or more proteins. Each protein can be referred to as the monomer in this context. Protein aggregation is also known as the non-native aggregation process. It is possible for the monomer to be a single folded chain, multiple protein chains that are disulfide linked to one another similar to how it is in a natively multimeric complex, as well as monoclonal antibodies (Roberts, 2015).

The risk of aggregation is one of the most difficult aspects of generating, delivering, and storing these protein therapies because they decrease the therapeutic's efficiency by lowering its concentration and facilitating its removal and has been proven to enhance immune response activation (Lundahl et al., 2021), and may further affect mode of delivery (Secher et al., 2022).

It is well established that nucleation is the initial condition necessary for the occurrence of the vast majority of the solution phenomena of the aggregation of protein. Therefore, the reduction of the number of soluble aggregates in the bulk of the product (drug) is an advantage to support in the product's long-term stability (Hua and Weiss, 2004). Different domains for monoclonal antibody, may denature irreversibly and independently through different route depending on the denaturation conditions, the structure of the denaturation found can be affected by the methods of denaturation. For example, the Fab domain and the Fc region respond more to heat, and pH respectively (Hua and Weiss, 2004). Research conducted by Chi et al (2015) on the physical degradation challenges of protein in aqueous solution, suggest that aggregation could be controlled by colloidal and conformational stability. In that regard, selecting suitable

conditions could be carried out to enable conformation stability and prevent intermolecular forces (Das et al., 2020).

Exposure of proteins to gas-liquid interfaces, formulation and filling can lead to denaturation. Adding non-ionic surfactants to protein formulations helps minimise aggregation and adhesion to surfaces, which can reduce drug activity. Brij 35, Pluronic F-68 and Tween 80, though not stable against heat stress, may induce the aggregation of human growth hormones. A molecule's stability can be jeopardised by an increase in concentration of surfactant (Joseph, 2006). However, Tween 80 is used in protein-based formulations and antibody formulations for the prevention of aggregation (Singh et al., 2017). It is also widely used in formulation of protein during various processing conditions for prevention of agitation-induced aggregation due to the effective nature in considerably low concentration. It further has an ability to inhibit aggregation, protein surface adsorption and hence act as a stabilizing agent against protein aggregation (Agarkhed et al., 2013).

1.3.4.1 Causes of Protein Aggregation

Conditions like temperature and shaking cause protein aggregation (Wang and Robert, 2018), Different factors affecting protein aggregation were studied by Kent et al, temperature and pH variations were found to affect protein unfolding resulting in increased aggregation of protein (Kent et al., 2018).

1.3.5 Overcoming Protein Instability

A protein medication needs to be properly stabilised to express its therapeutic characteristics. Lack of stabilisation may have negative consequences for both safety of patient and product efficacy (Bawa et al., 2019). To ensure patient safety and stability conditions, the physical

and chemical factors that contribute to protein instability must be addressed (Butreddy et al., 2021).

The development of formulations focuses mainly on recognizing major changes for example aggregation, deamidation, fragmentation and oxidation as every such degradation kinds reduce the bio effectiveness or efficacy of the drug and produce adverse immunological effect of the drug, A shelf life of more than 12 or 24 months is vital for the proteins to be of commercial value (Tian et al., 2007; Falconer et al., 2011). Developing stable therapeutic proteins requires a variety of steps, including careful handling, appropriate manufacturing processes, appropriate excipient selection, and optimal storage. A critical evaluation of the impact of each factor on the stability is evaluated to reduce chemical and physical losses due to different forms of instabilities (Wang, 1999). For example, several proteins have been shown to be stabilised against aggregation when formulated with sugars.

Antitrypsin-α1,JFN-ϒ, basic fibroblast growth factor, and IFN-βare only a few of the proteins that contain soluble aggregates, and HP-SEC (High Performance Size Exclusion Chromatography) may detect them. Tolerable quantities of soluble aggregates are acceptable in medicinal goods provided that they do not become insoluble over time.

An alteration in the therapeutic protein's immunogenicity may be caused by an increase in soluble aggregates. For example, insulin and IL-1β can be viewed as aggregates. FTIR, Raman, and electron spin resonance spectroscopy, as well as light scattering techniques (UV absorption), can all be used to find insoluble clumps (Joseph, 2006). Stabilisers are the most significant class of excipients because they protect biological molecules exposed to lyophilisation from the chemical and physical instability that can result from the process. Stabilisers can help to avoid these changes from occurring during drying (Bjelosevic et al., 2020). Addition of additives and excipients may protect molecules in protein from chemical and physical degradation as they help to keep adequate hydration around protein molecules whilst avoiding denaturation at the same time (Hawe and Freb, 2007). Also, they are

introduced to preserve the stable and folding reversibility of proteins. Reduced adsorption can also be attained by addition of surfactants or the use of special containers (Hawe and Freb, 2007).

Furthermore, amino acid seems to be the most favored excipients because they are naturally present in the blood with low toxicity (Bjelosevic et al., 2020) as they have been proven in the formulation of proteins, examples include arginine, histidine, glycine, glutamate. They have been used commercially in protein therapeutic products as stabilizers (Falconer et al., 2011), other amino acids such as alanine, proline, lysine and serine have also been studied. The study of arginine was discovered to prevent protein aggregation by protein affinity chromatography during purification (Ejima et al., 2007) and enhance the folding yield in (in vitro) protein. The mechanisms for arginine's activity have not been fully explained. Nonetheless, arginine increases the equilibrium solubility of folded and unfolded proteins and further enhances the stability of monoclonal antibody. Studies by Carpenter et al (1986), showed amino acids can be used as cryoprotectant in the formulation of protein and lyoprotectant in the prevention of aggregation, dehydration-induced unfolding of protein (Tian et al., 2007). Trehalose and some sugars also act as cryoprotectant. Wang et al (2006) showed the lowered viscosity of salt and amino acid on high concentration solutions of two different monoclonal antibodies, in the study it was observed that monoclonal antibody displays colloidal solution in highly concentrated formulations like aggregation, low stability and high viscosity. It is known that these properties prevent delivery in subcutaneous routes (Wang et al., 2006). Formulation of dosage form can be affected by extreme conditions like freeze drying and spray drying, causing problems with stability and bioactivity of protein (Cleland and Carpenter, 2001).

1.4 Stabilization by Drying Technique

Proteins can be produced in dried forms of formulation to overcome instability through different techniques; either by lyophilization (freeze dry) or spray drying. It can exist in different solid forms such as amorphous, crystalline or a mixture of amorphous and crystalline before and after being lyophilized. These proteins display different biological activity, solubility, chemical and physical stability during processing in extreme conditions.

1.4.1 Spray drying

Drying of protein is the process of extracting water from liquid protein turning them into solid powder form for prolonged storage stability (Elkordy et al., 2002). Spray-drying is a process where the sample is prepared first as a liquid, then atomized in a drying chamber and the drying of the droplets takes place using hot air or high temperature (Fourie et al., 2008; Lee et al., 2011). In the drying chamber, the solvent in the solution droplets evaporates and the dissolved chemicals precipitate, resulting in particle formation (Sou ta al., 2011). Table 1.1 shows a list of approved spray dry medications. One of the challenges is the destabilization of the production process because of hot temperature and pressure (Ramezani et al., 2014). Excipients are therefore used in the manufacture and development of the medicine to achieve maximum stability, prevent aggregation and reduce the chemical degradation process to the barest minimum. During this process, selecting the appropriate agent for stabilization has become a challenging task because of the approval of a few excipients for use by the regulatory bodies (Challener, 2015). However, there is still improvement and development of new excipient to alter bioavailability and make formulations that are economically acceptable.

Shoyele and Cawthornea, (2006) researched on spray drying with findings that during spraying, proteins may lose their biological activity due to protein adsorption at the air-liquid interface. Therefore, surface active substances like surfactants can be used to stabilize the

different proteins during spraying via reduction of the effects of interaction between the air-liquid interfaces.

Table 1.1: Spray-dried protein pharmaceuticals commercially approved by FDA and EMA (Pinto et al., 2021). FDA. Food and drug administration, EMA. European medicine Agency

Product	Active pharmaceutical ingredient	Formulation	Excipients	Route of administration	Approval
Exubera® (Pfizer) (Discontinued)	Insulin	Ready to use powder	Mannitol, glycine and sodium citrate	Inhalation	Jan-2006
Trelstar® LA (Verity Pharmaceuticals)	Triptorelin pamoate	Microsphere suspension (powder for reconstitution)	PLGA, mannitol, carboxymethyl - cellulose, polysorbate 80	IM injection	Oct-2010
Somatuline® LA (Ipsen)	Lanreotide acetate	Microsphere suspension (powder for reconstitution)	PLGA, mannitol, carmellose sodium, polysorbate 80	IM injection	Nov-2013
Raplixa® (The Medicines Company)	Fibrin sealant (human)	Ready to use powder	Trehalose, calcium chloride, albumin, sodium	Topical	Apr-2015

			chloride, sodium citrate, L-arginine hydrochloride		

There have been several researches conducted on spray drying techniques. Hulse et al (2008) compared the effect of spray-dried multiple and single excipient, blended into the protein system, with lysozyme as the model of protein and trehalose, mannitol and sorbitol as the excipient. The protein samples were stored for a month at 75% relative humidity and temperature of 40^{o}C. The result showed that spray-dried proteins were more stable when compared to unprocessed protein after storage and none of the protein excipient formulation showed a 100% biological activity, instead lysozyme spray-dried in the absence of excipient showed an increase in biological activity. He further discussed that protein containing trehalose was preserved after spray dry while spray-dried lysozyme with a combination of mannitol and trehalose maintained a biological activity compared to mannitol and sorbitol.

Niogain et al (2012) researched on the production of micro sized particles that are suitable for inhalation through spray drying however, another model of protein known as trypsin was used. In the experiment, the solvent system was adjusted for adequate protein solubility. Trypsin was spray-dried with raffinose from a methanol: n-butyl acetate solvent system. There was a readjustment of the solvent system with the inclusion of water to trypsin and co-sprayed with raffinose hydroxypropyl-β-cyclodextrin and trehalose. They concluded that production of protein micro-particles for inhalation was best to select the proper excipient with the adjustment of the solvent system.

Spray-drying technology has been effectively employed in the production of a few protein medicines over the last 15 years. Exubera® by (Pfizer), an inhaled insulin powder, was the first commercial spray-dried protein hormone when it was introduced in 2006, though later

withdrawn. In 2010 and 2013, Poly (lactic-co-glycolic acid) (PLGA) microspheres and lanreotideacetate respectively were approved. Raplixa® (ProFibrix BV) was the first protein medicine to be licensed in the United States and was made using aseptic spray-drying technology in 2015. Other protein medicines such as those made by spray-drying are currently in clinical development in various forms of dosages (Vehring et al., 2020; Pinto et al., 2021).

1.4.2 Electrospray Technique

In the year 1600, Williams Gilbert made the first observation and recording of electrospray, which was also known at the time as Electro Hydrodynamic Atomization (EHDA) (Xie et al., 2015). It is a technique in which electrical forces cause a liquid jet to fragment into droplets. For the generation of nanoparticles, one can achieve a variety of spraying modes by adjusting both the magnitude of the electric stress and the kinetic energy of the liquid as it exits the nozzle. Using electric forces, this tested method may produce extremely minute droplets of a monodispersed size from a liquid source (Xie et al., 2015). On the other hand, it has the potential to slow down or even stop the degradation of protein drugs and to offer precise control over the morphology of the particles and their size distribution. Electrospray has the potential to achieve controlled monodispersity and morphology of particles without causing denaturation of bioactive molecules at any point in the process via electric charges.

The theory of charged droplets is the foundation of the electrospray process. This theory "states that an electric field applied to a liquid droplet as it exits a capillary is possible to deform the interface of the droplet" (Bock et al., 2012). Electrospray according to some published literatures, is superior to other drying techniques because it does not require a high temperature, and as a result, it may use little no emulsifier or may require additional drying. Xie et al., (2008) conducted a study on the encapsulation of protein therapeutics in

biodegradable microparticles by coaxial electrospray using lysozyme as a model protein. According to the findings of the paper, electrospray has the potential to be an effective method for encapsulating biomolecules like proteins. Bock et al., (2012) also conducted a review on electrospray of polymer with therapeutic molecules as a state of the art. Based on the findings of this review, the electro spraying technique has the potential to become a promising method for the production of particles that encapsulates therapeutic molecules and have the potential to release those molecules as the particles erode. Insulin was one of the first biomolecule to be utilized for electrospray process (Prin et al., 2017). Gomez at al observed that decreasing the concentration of insulin solution or by reducing the flow rate of the electrospray system and smaller particles can be generated.

1.5. Stabilization by Addition of Excipients

Excipients and active pharmaceutical ingredients (API) are both components of finished pharmaceutical preparations. The inclusion of excipients is one of the most common methods used in the stabilization of liquid formulation (Falconer et al., 2011), as they influence and interact with the stability structure of protein. Table1.2 outlines the numerous classifications and roles that can be assigned to excipients based on the part that the formulation requires them to play, and this information can be found in the table.

Table 1.2: Examples of excipients used in pharmaceuticals for protein formulation (Chi, 2012; Karmerzell et al., 2011).

Categories	**Uses**	**Example**
Preservatives	Preservation against microbial growth.	Phenol, M-Cresol, Benzyl Alcohol
Buffering agent	Used in the control and maintenance of pH.	Acetate, Tris, Phosphate, Histidine, Citrate.

Surfactant	For control of surface adsorption and inhibition of protein surface denaturation	Polysorbate 20 & 80.
Carbohydrate and Sugar	As bulking agent for lyophilisation, protein stabilisers, tonicifying agent	Trehalose & Sucrose (also known as cryoprotectant) Sorbitol, Lactose, Glucose, Mannitol
Polymer	As bulking agent for lyophilisation, also to increase solution viscosity, also for drug delivery vehicle.	Gelatin, Poly Lactic-Co-Glycolic Acid (PLGA), Polyethylene Glycol (PEG)
Salt	For stabilization and destabilization effect on protein, osmolarity and for physiological tonicity.	Sodium Sulphate, Sodium Chloride, Potassium Chloride.
Specific Ligands	Binding of protein and conformational stability against stress-induced folding.	Ligands, Amino Acids, Polyanions, Metals.
Anti-oxidants and Chelators	Scavengers of free radicals that bind metal ion.	Amino Acid (Methionine and Histidine), Ethanol, Ethylenediaminetetraacetic Acid (EDTA), Diethyltriaminepentaacetic Acid DTPA

Amino Acid	Used for specific protein interactions also as antioxidant for some protein	Arginine, Histidine, Proline, Lysine, Methionine, Glycine.

Excipient plays a variety of functions in the pharmaceutical dosage form such as assisting the active ingredients in the maintenance of preferred conformations. It helps in the maintenance of pH and osmolarity of liquid formulations. In addition to this, it has a substantial impact on the bioavailability. Excipients have the potential to have a significant impact on the physiology of the gastrointestinal (GI) tract, which can result in alterations to the GI motility as well as the permeability of the membrane. However, there are no protein medications currently administered via oral route. For instance, mannitol and sorbitol both impact the amount of time it takes for food to pass through the intestinal tract. This can present some difficulties when combined with medications that have a low permeability (Ubic consult, 2014).

Immunoglobulin (monoclonal antibodies) was studied with and without Tween® 80 by Kapp and colleagues. Tween® 80's potential to block the adsorption of monoclonal antibodies to hydrophobic interfaces was investigated in more detail, there was no difference in the adsorption behavior between the antibodies, but the presence of surfactant at 30 mg/L reduced the behavior. They further resolved that the sequence in which the surfactant and protein molecules are exposed to the surfaces has a significant impact on the amount of protein adsorption (Kapp et al., 2015).

Investigation was also conducted by Kanthe et al (2020) on the competitive adsorption between surface-active excipients and antibody proteins, and then the concentration of surfactants required to prevent mAbs from adsorbing at the interface (air-water) his finding showed the adsorption of monoclonal antibodies (mAbs) to the surface and surface domain was inhibited at high concentrations of surfactants also, competition between excipients for the interface is directly correlated with the mAb surface activity (Kanthe et al., 2020).

Among the additives that have been examined in the past are cyclodextrin (reviewed by Branchu, 1999, Cooper, 1992, and Irie and Uekama, 1999), polyols (reviewed by Gekko, 1982 and Wimmeret al., 1997), sugar (reviewed by Back et al., 1979, Tanaka et al., 1991, and Uedaira and Uedaira, 1980),

1.5.1 Excipients used for this Research.

1.5.1.1 Pluronic F-127

Pluronic powder F-127, CAS 9003-11-6, purchased from Sigma Chemical company, Pcode: 101561723, CMC. 950-100PPM (-25ºC). It is a non-ionic surfactant polyol with molecular weight of approximately 12,500 Daltons with a chemical formula of $H(OCH2CH2)_x(OCH2CHCH_3)_y(OCH2CH2)_x$ OH (Figure 1.4) It facilitates the solubilisation of water- insoluble dyes and other materials in physiological media.

It is also known as poloxamer 407 found in the class of synthetic block copolymer consisting of hydrophobic poly propylene oxide (PPO) and hydrophilic polyethylene oxide (PEO), they can be found either in liquid, solid or paste form. Pluronic possess properties of surfactants which helps them to interact with hydrophobic surfaces and biological membranes as a result of their amphiphilic characteristics making it a polar water-soluble hydrocarbon chain. Amphiphilic block copolymer (unimers) molecules are self-arranged into micelles. (a packed chain molecule) in aqueous solutions (Shirwaiker et al., 2014). Micelle formations are dependent on temperature and can affect a biomaterial degradation property below a certain temperature characteristic called the critical micelle temperature where both the ethylene and propylene oxide blocks are hydrated, and the poly propylene oxide block becomes soluble. (Bearat and Vernon, 2011) Various research have been conducted with different types of pluronic, Wang and Johnson (1993) researched on the “enhanced stability of two model proteins in an agitated solution environment using poloxamer 407”, it was observed that the

concentrations applied had positively affected the biological activity of the protein. Another specie/ source of pluronic was applied on the conformational stability of a protein named salmon calcitonin in lyophilized solid form and aqueous solution. It was concluded it was a preferable surfactant for the prevention of secondary structure changes in aqueous solutions at 40°C (Lee and Lin, 2011). Adsorption of antibody into pluronic nanoparticles was investigated by Gomez et al (2011), it was concluded that pluronic improved conformational change undone by protein.

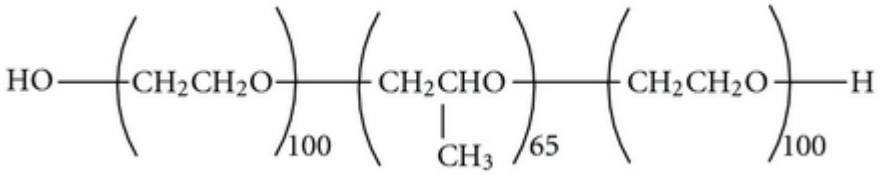


Figure 1.4: Structure of Pluronic F-127 (Butt et al., 2012)

1.5.1.2 Beta Cyclodextrin

Beta cyclodextrin powder by Sigma Aldrich (C4767-25G) LOT#MKBX4220V, Pcode: 1002389461, CAS-7585-39-9, melting point: 290-300ºC (dec)(lit) Molecular weight: 1134.98 g/mol. Beta cyclodextrin (β-CD) is a type of cyclodextrin (CD), a cyclic oligometric sugar-based molecule (oligosaccharides), a torus shaped with the look of a cone of an ice cream. Other types of CD include the alpha and gamma glucose unit. They are non-toxic, chemically stable molecule with hydrophilic surface and hydrophobic cavity as shown in Figure 1.5. Inclusion complexes can be formed in cyclodextrins with many hydrophobic compounds (Fernandez et al., 2002). This makes it valuable in the cosmetic, food industries and approved for pharmaceutical applications. (Haynes, 2009) The cavity of the cyclodextrin is lined by hydrogen atoms and the glycosidic oxygen bridge, respectively. A high electron density is produced as a result of the glycosidic oxygen bridges' non-bonding electron pairs being guided

toward the inside of the cavity. This electron density borrows some properties from Lewis bases. One hydrogen is formed when the C-2-OH group of one glucopyranoside unit combines with the C-3-OH group of the adjacent glucopyranose unit. In the case of the CD molecule hydrogen bond is formed from a complete secondary belt, as a result, the beta cyclodextrin is rather described as structurally rigid. The intramolecular hydrogen formation is most likely explained as the observation that β-CD has the lowest water solubility of all the CD (Szejtli, 1998). Cyclodextrins are tiny molecules that have a hydrophobic core and a hydrophilic exterior. Because of this, cyclodextrins are ideal substrates for interacting with other molecules (Anand and Mukherjee 2013). There is a lot of disagreement in the literature regarding the mechanism of protein stabilisation against mechanical agitation seen with cyclodextrins. Many researchers claim that the primary mechanism underlying stabilisation is the binding of cyclodextrins to hydrophobic residues (Frokjaer and Otzen, 2005; Hartl et al., 2013). Studies conducted by Hartl et al., 2013 using HP cyclodextrin on antibody stability concluded that the interaction of high HP cyclodextrin reduced the thermodynamic stability of antibodies at high and low concentration leading to a decrease in monomer after thermal stress, their study went further to demonstrate that HPCD interacts directly with proteins rather than stabilising proteins through a combination of polyol and surfactant-like characteristics. Loftsson, 2021 reviewed cyclodextrin in parenteral formulation and concluded that CDs are solubilizing complexing agents that are frequently employed in high concentrations in a variety of commercial parenteral solutions. The water-soluble beta cyclodextrin derivatives, HPbeta cyclodextrin and SBEbeta cyclodextrin, are quickly eliminated in the urine unmetabolized, thus CDs often have little impact on the pharmacokinetics of a given medication. They are typically chosen over other solubilizing excipients like surfactants and organic solvents in parenteral medication formulations because of their extremely good toxicological and pharmacokinetic properties.

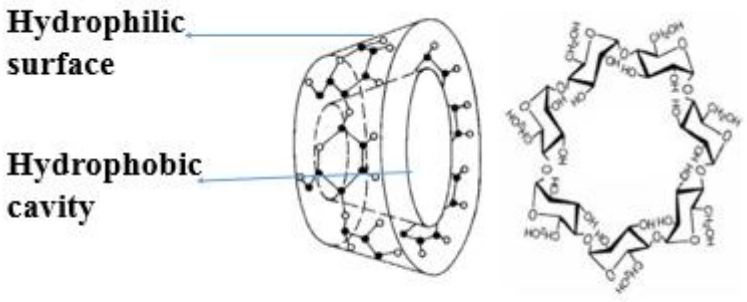


Figure 1.5: Structure of Betacyclodextrin molecule (Haynes, 2009)

1.6 Thermal Analysis of Protein

It is a thermo-analytical approach that uses the difference in the heat amount needed to raise the temperature of a reference and sample as the temperature is measured in a controlled environment, it also measures the temperature associated with the material transition as a function of temperature and time, the measurement further gives quantitative and qualitative changes, chemically and physically involving the change in heat capacity forming endothermic and exothermic reactions (Intertek online, 2012). It is a useful approach for determining the physicochemical properties of most substances. Thermal analysis is a technique used to detect denaturation in protein. DSC is a technique of analysis used in the pharmaceutical industry. (Perkinelmer online, 2014) defines DSC as a thermal analysis technique that observes the change in materials measurement of temperature and heat; during this process, a sample of a known mass is heated or cooled and the changes in its heat capacity are tracked as changes in the heat flow allowing the detection of transition such as phase changes, glass transition. DSC is used in various industries: manufacturing, agriculture, electronics and pharmaceutical.

It's a common method for investigating the thermal behaviour of tiny and big molecules, it is also used for the investigation of thermal stability and the reversibility of folding in protein solutions (Elkordy et al., 2008). The enthalpies and transition temperature are the determining factors. It is used to compare and determine the existence of the distinct phase limitations (Schulz, 1998). Protein conformational stability varies, for example, monoclonal antibody conformational stability is observed to be higher in research papers compared to other proteins. It is usually hard to achieve the complete unfolding of the molecule at a temperature as high as 100^{o}C. This is often the technique of choice when measuring the thermodynamic parameter and folding reversibility of several types of protein. DSC profile of antibody molecule should have three peaks identified as Fab (fragment antigen binding site), CH_2 and CH_3 domains. Razinkov et al., (2015) states that under typical formulation conditions, the most stable domain is usually the CH_3, and CH_2 is the least stable. Depending on the factors of the formulation, each domain melting temperature can be shifted because they are not constant. It is generally observed that there could be an increase or decrease in T_m due to the presence of salt and pH. Also, the T_m can also be affected by excipients. Understanding the molecular mobility and the reactions of chemicals in protein is very challenging in the prediction of stability and storage of any medicine.

1.7 Model of Protein

Three types of protein will be studied in the research, Protein as:

(a) An enzyme (**lysozyme**): - As thousands of biochemical reactions are performed daily to function, protein enzymes help to lower the activation energy of many reactions, assisting them to function faster than they would in a normal environment. An example is lysozyme, an enzyme naturally found in bodily secretions such as tears and saliva. They usually act as antimicrobial agents via its cleavage to the peptidoglycan component of bacterial cell walls leading to cell death (Oliver and Wells, 2015).

(b) As a hormone (**Insulin**): - They coordinate activities and send signals through the body. Insulin regulates glucose and signals the cells throughout the body to absorb the sugar and use it for energy.

(c) As an immunity (**Monoclonal antibody**): - They help the body fight against infection. For each antigen that enters the body, there is a distinct and separate antibody that fights intruders.

1.7.1 Lysozyme

Lysozyme is used by ENT surgeons for the treatment of canker sores and sore throats, also used in ophthalmology for eye drops solution and decontaminating contact lenses. (Heyns, 2001). The presence of lysozyme in organisms and their related biological fluid isn't by chance because it plays an important part in the immune systems. Lysozyme, being an over-the-counter drug is used to increase the natural defence of the body against bacterial infection. It is a type of protein that is a component of the immune system and may be found in secretions such as tears and saliva. These proteins have the ability to function as antibiotics and breakdown the cell walls of some types of bacteria. Lysozyme is created in the secretions of mucus and tears to protect against the invasion of bacteria in those places. Lysozyme is also present in blood circulation to assist in preventing bacteria from moving to other parts of the body.

It is believed that Sir Alexander Flemming, discovered lysozyme first and later developed penicillin, making it the first antibacterial substance ever discovered. The process was kicked off by placing a drop of mucus on a plate containing bacteria. During this process, it was determined that the bacteria were destroyed by the mucus. In addition, the size of the lysozyme molecule prevented it from being able to penetrate cells. According to the available evidence, lysozyme was the first enzyme to have its three-dimensional structure figured out by Prof

George W Kenner (Vocadlo et al., 2001; Farlex, 2023. Lysozyme has been chosen for this research because it has been studied and characterized in different literatures (Elkordy at al., 2008; Ji et al., 2016; Mutukuri et al., 2021) and it is a good model of protein for testing excipient effect after spraying

1.7.1.1 Structure of Lysozyme

The primary biochemical role of lysozyme is to split the B-1,4 glycosidic bond between N-acetylmuramic acid and N-acetyl-D-glucosamine. It is one of the most powerful proteins, with approximately 129 amino acids in its composition. (Figure 1.6) with a high ionic strength and pH. The enzymes are dependent on the tertiary structure for maintaining their biological activity (Ghaderi and Carlfors, 1997; Farlex, 2023).

Many bacterial species possess the bonds in their cell wall providing lysozyme with antibacterial properties.

Lysozyme is also known as muramidase because of its ability to cleave the bond that joins N-acetylmuramic acid to the sugar molecule that is immediately adjacent to it. The enzyme is referred to by its scientific name, which is N-Acetylmuramide glycanhydrolase.

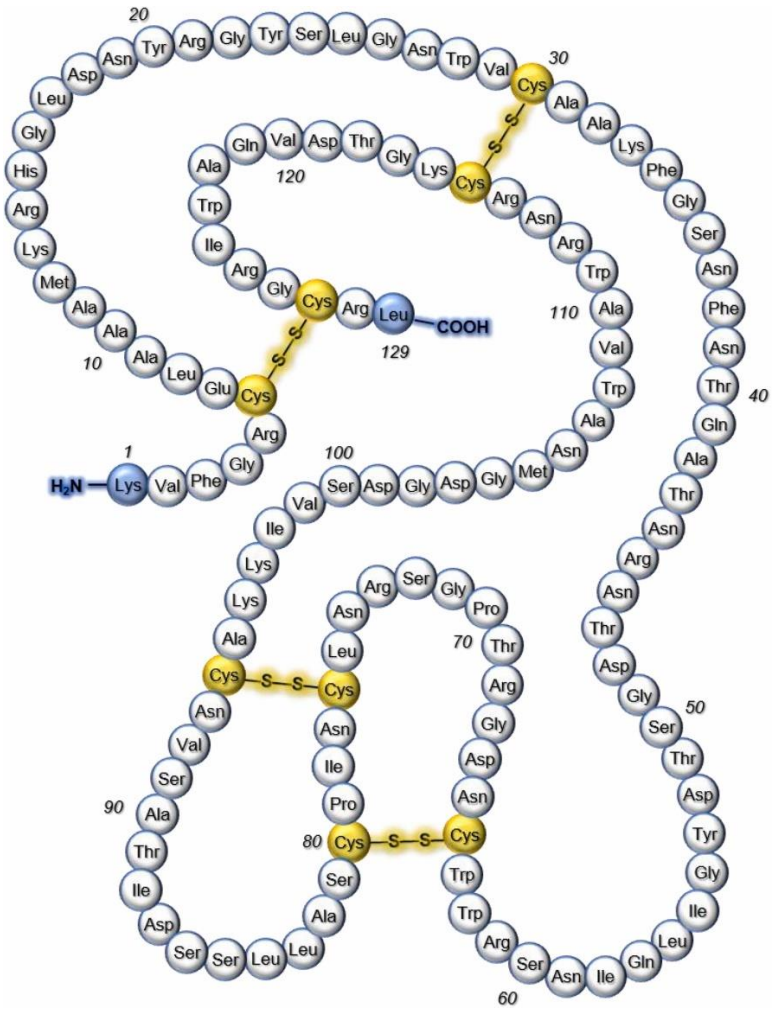


Figure 1.6: Structure of Lysozyme. Melinte et al., 2021

Since the purpose of an enzyme is to increase the rate at which a chemical reaction occurs in the body, the antibacterial properties of lysozyme come from the enzyme activity it carries out. In most cases, polysaccharides can be discovered in the cell walls of the bacteria that are affected. These polysaccharides have amine groups (NH_2) in addition to sugars and sugar side chains in them. When water molecules are added to the sugar linkage, it triggers lysozyme to begin degrading the polysaccharide, which in turn causes the polysaccharide to rupture open. Ionic strength and pH both play a role in determining how active it is. It is effective at a pH range ranging from 6.0 to 9.0. At a pH of 6.2 and an ionic strength of 0.02 to 0.1M, the enzyme reaches its highest level of activity (Aldrich, 2012).

1.7.2 Insulin

Insulin is a type of hormone that is created in the pancreas for the purpose of regulating blood sugar. Throughout the history of science, insulin has been one of the molecules that has been subjected to the most research. Its most prevalent application is in the medical management of a condition known as diabetes mellitus (DM). Frederick Banting and Charles Best, who were manufacturing bovine and porcine insulin at the time, were the ones who made the discovery in 1921 (Wintersteiner et al., 1928). Even though the first batch of insulin had unfavourable side effects and was relatively impure, it vastly improved the quality of life and outlook for many diabetic patients. Despite these drawbacks, it was a significant step forward. Today, further development in production of insulin has led to highly purified products and has further improved the safety and efficacy of insulin therapy. Insulin has been available since 1925, Canadian researchers first observed responses physiologically to animal insulin injection in patients with type 1 diabetes in 1922, insulin was the first protein to be fully sequenced. It was initially extracted from pork and beef pancreas, and in the 80s, human insulin was produced synthetically with the help of recombinant DNA technology. In the United States of America, synthetic human insulin replaced the beef and pork insulin and presently, insulin analog is currently replacing human insulin (Kennedy, 2019).

Table 1.3. Summarizing the types of insulin in the market with the peak, duration, onset time and role in blood sugar management. (Kennedy 2019; British pharmacopeia 2019)

Type of Insulin and Brand Name	**Peak**	**Duration**	**Onset**	**Role in Blood Sugar Management**	**Appearance/Shelf-life**
Rapid-Acting					
Aspart ® (Novolog)	40-50min	3-5 hours	10-20mins	Rapid-acting insulin covers insulin needs for meals eaten at the same time as the injection. This type of insulin is often	White or almost white powder/ 30months

				used with longer-acting insulin.	
Lispro® (Humalog)	30-90min	3-5 hours	15-30mins		White or almost white powder/3 years
Glulisine ® (Apidra)	30-90min	1-2 1/2 hours	20-30mins		Clear colourless aqueous solution/2 years
Long acting					
Insulin detemir® (Levemir)	6-8 hours	up to 24 hours	1-2 hours	Long-acting insulin covers insulin needs for about one full day. This type is often combined, when needed, with rapid- or short-acting insulin.	Clear/ 30months
Insulin glargine® (Basaglar, Lantus, Toujeo)	No peak time, insulin delivery at steady level	20-24 hours	1-1 1/2		Clear solution / 2 years
Insulin degludec® (Tresiba)	No peak time	42 hours	30-90mins		Clear colorless neutral solution/30 months
Short acting					
Velosulin® (for use in the insulin pump)	1-2 hours	2-3 hours	30mins - 1 hour	Short-acting insulin covers insulin needs for meals eaten within 30-60 minutes.	
Novolin ®	2-5 hours	5-8 hours	30mins-1 hour		
Pre-Mixed					
Humulin® 50/50	2-5 hours	18-24 hours	30 mins	These products are generally taken two or three times a day before meal time.	Sterile suspension of a white crystalline precipitate of isophane human insulin in an isotonic phosphate buffer/ 3 years
Humulin® 70/30	2-4 hours	14-24 hours	30 mins		

Humalog mix® 75/25	30 mins -2 1/2 hours	16-20 hours	15 mins		
Novolin® 70/30	2-12 hours	up to 24 hours	30mins		
Novolog® 70/30	1-4 hours	up to 24 hours	10-20mins		
Intermediate acting					
NPH (N) ®	4-12 hours	18-24 hours	1-2 hours	Intermediate-acting insulin covers insulin needs for about half the day or overnight. This type of insulin is often combined with a rapid- or short-acting type.	cloudy

A formulation of insulin lispro listed in Table 1.3, is a type of rapid-acting human insulin analog used to improve glycemic control in children aged 3 and older as well as adults with type 1 diabetes and adults with type 2 diabetes (Voelker, 2018).

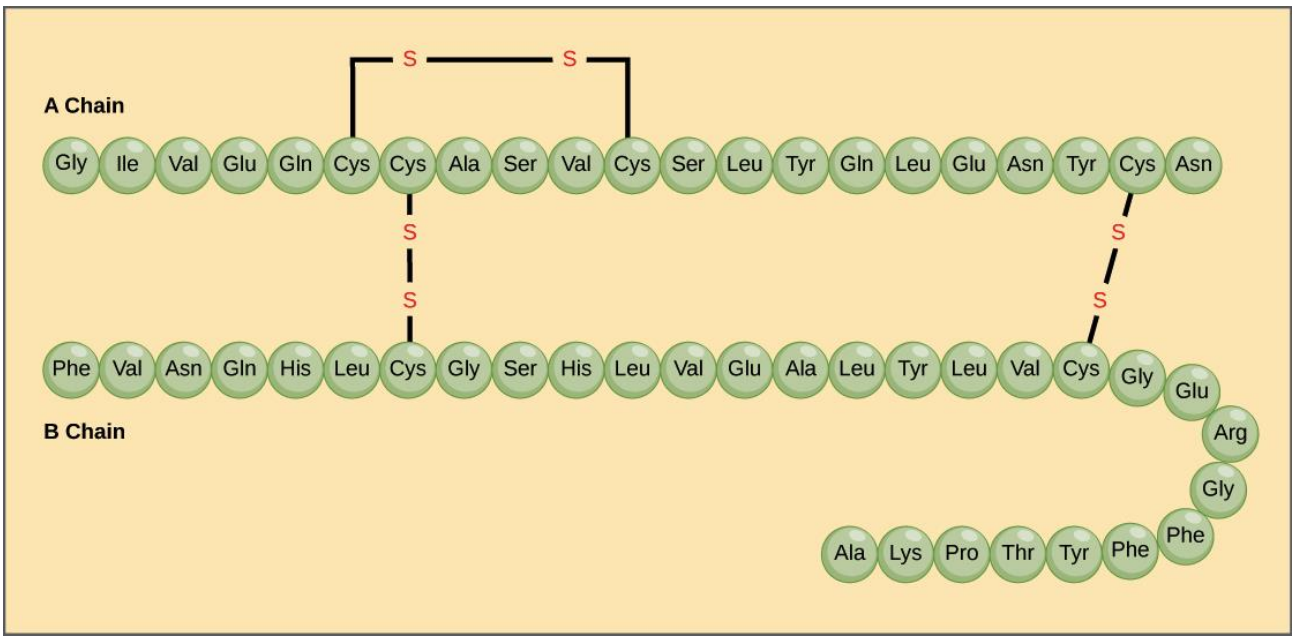
Insulin was sourced from beef and pork but are no longer used commercially in the United States, though importation of insulin is still allowed via personal importation for patients who cannot be treated with human insulin. The drugs are produced in form of vial, pen and pumps and administered subcutaneously via an insulin syringe, insulin pen or pre-filled pen device. They can also be administered as an insulin infusion using a wearable personal insulin pump or through an intravenous insulin infusion.

According to Pfizer, their product Exubera, which was released in the beginning of 2006, was the first dry powder, inhaled insulin accessible for the treatment of diabetes and was approved by both the American and European Drug Agencies (FDA and EMEA). The advent of inhaled dry powder insulin brought with it the promise of a bright future for the development of further improvements in pulmonary insulin delivery. In patients with type 1 diabetes, the inhaler was used in combination with basal insulin. In patients with type 2 diabetes, the inhaler was used

in combination with basal insulin and oral treatments. The inhaler was not without its drawbacks. Some patients had a difficult time following the dosage instructions because the usage of many blisters necessitated numerous inhalations. (Neumiller et al., 2007). Unfortunately, in October 2007, Pfizer announced it was taking the drug off the market stating that the drug failed market acceptance. Since 1924 there have been many failed attempts to get away from insulin injections. The three-alternative method of delivery mainly focused on include delivery to the deep lungs, the upper nasal airways, and through the stomach which oral-lyn ® and afrezza® achieves (Siekmeier and Scheuch, 2008).

1.7.2.1 Structure of Insulin

Insulin molecules is a peptide consisting of an A chain of 21 amino acids, a B chain of 30 amino acid, all together comprising 51 amino acids linked by two disulphide bonds between two cysteine residues, a disulphide bond is the linkage between two sulphurs Figure 1.7a. The amino acids are secreted by the β cells in the islets of Langerhans (Weiss et al., 2014). The clarification of the primary structure of insulin was established in 1955 after Sanger and co-workers carried out an extensive work on it. The bovine insulin molecule contains alanine instead of threonine and contains valine instead of isoleucine in eight and ten position of the A-chain respectively. Insulin molecules contain ionizable groups due to the size of amino acid residues capable of attaining a positive charge and ten amino acids capable of attaining a negative charge. The molecular weight for bovine and porcine insulin are 5734Da and 5778Da respectively (Weiss et al., 2014). Insulin may be called a polypeptide and a protein as a result of its structure and size.



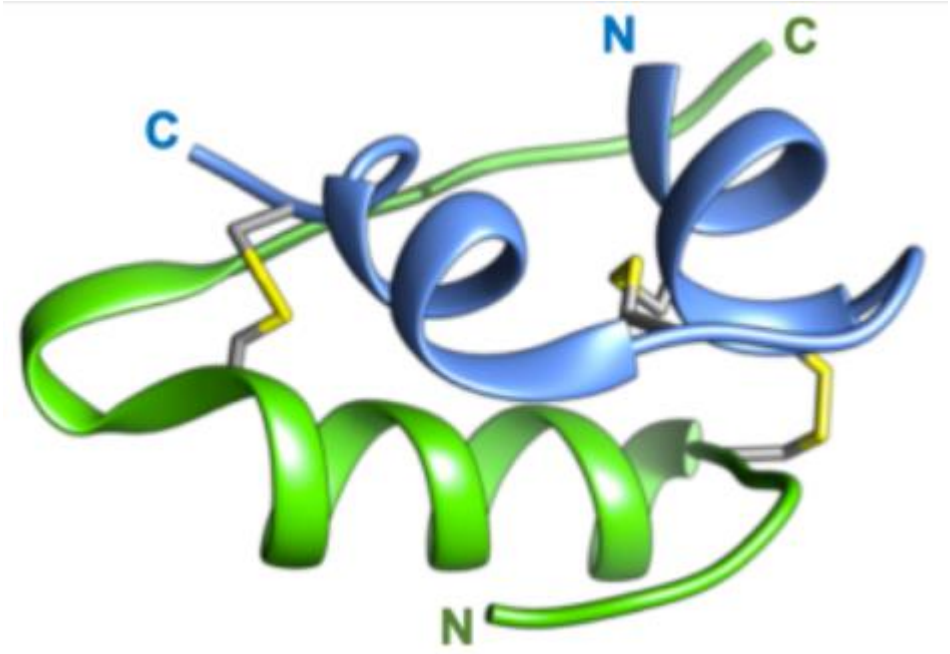


Figure 1.7(a): Primary structure of human insulin (lumen learning 2019) and a folded structure of insulin extracted from protein data bank.

The structure contains elements of stability, self-assembling, trafficking and receptor binding.

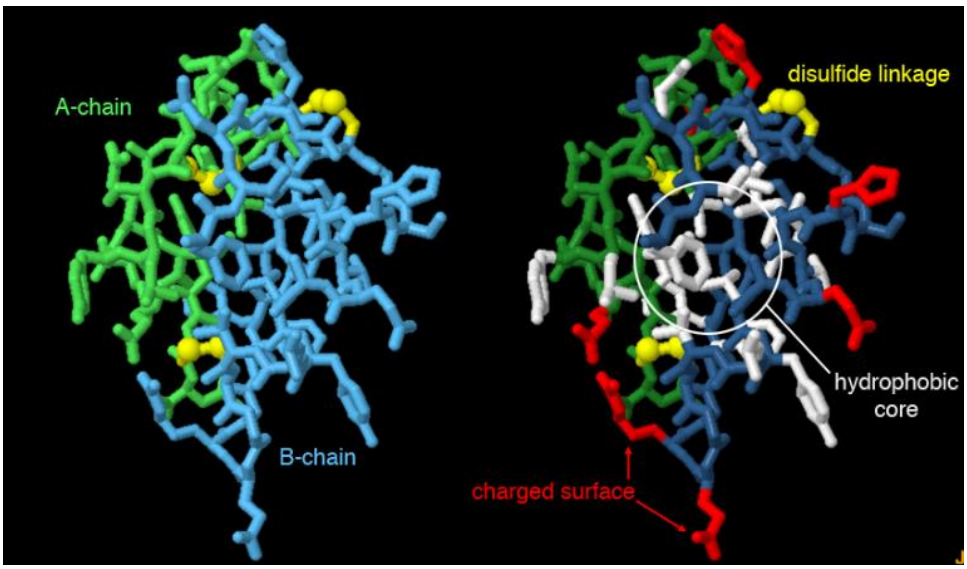


Figure 1.7(b): Explored structure of insulin (extracted from protein data bank)

An insulin monomer (made of an A-chain and a B-chain) is found in the hormone's hexameric complex, therefore it is important to note that hexamer comprises of 3 dimers hence contains 6 monomers Figure 1.8. The protein structure is stabilised by a number of key structural features, cystein amino acids are linked with three disulphide bridges (Figure 1.7 b) which help to keep the protein in its stable state. Charged amino acids such as arginine and glutamate cover the hydrophobic core formed by leucine and isoleucine, which is rich in carbon atoms. The hydrophobic core is surrounded by a layer of charged amino acids like arginine and glutamate with favorable interaction with the water surrounding it (Ward and Lawrence, 2011).

Insulin is present at very low concentration in the bloodstream, known as monomers, in its biologically active form, they assemble into dimers at higher concentrations and into hexamers. At neutral pH and in the presence of zinc ions, hexamers are formed when each of two (2) zinc ions bind to its residue. To form insulin crystals, summation of about ten (10) zinc ions binds in the other side forming insulin crystal. When there is release of insulin into the blood stream, there is dissociation of hexamer into monomers which then binds to the insulin

receptor. In pharmaceutical formulation, hexamers are the preferred form of the hormone because of its resistance to degradation and fibrillation (Lisi et al., 2014).

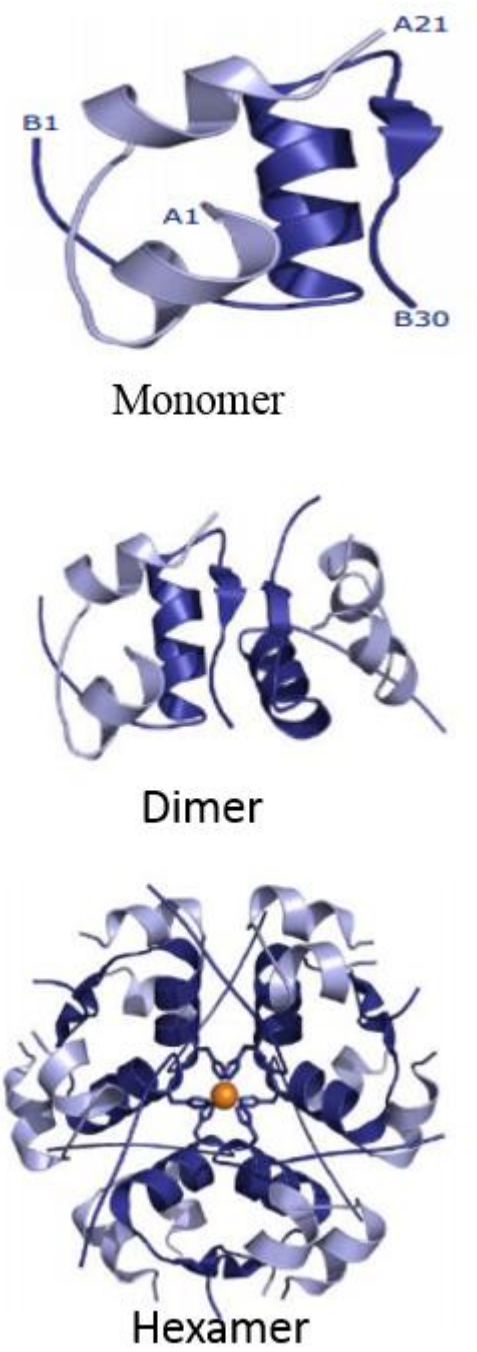


Figure 1.8: Illustration of the self-association form of insulin. (Bhatnagar et al., 2006)

1.7.2.2 Insulin Stability

Insulin is very sensitive to extreme cold or hot temperature, indoor light, and sunlight. It should not be used when subjected to an environment that is extremely hot or cold. Drug manufacturers' advice that unopened insulin is best stored inside the fridge between 2-8 degrees Celsius and insulin that has not been opened and put in the fridge remains okay until the expiry date printed on the container of the insulin (Barros, 2018).

The main challenge of insulin stability is degradation which generally means a loss in potency. It could be during preparation, manufacturing, transportation or storage. Degradation can be in the chemical, physical or biological form.

Since insulin was introduced as a drug, the structure and stability have been subjected to series of studies, it has been hypothesised that the association state of insulin and the conformation of the hexamer, which is the most stable of the several types of insulin, play a significant role in the chemical and physical stability of insulin and other parameters. Insulin is said to be stored in the zinc and calcium rich vesicles as metal- stabilizing hexamer. Since it is common knowledge that insulins biological form can be destroyed by the process of boiling, a lot of research have been carried out to investigate the impact on insulin from heating.

These studies conducted have looked at insulin fibrillations and have been carried out in a solution that is acidic under specific conditions in which insulin is primarily in equilibrium with its dimers and monomers. After the NMR (Nuclear Magnetic Resonance) verified the initial result, which was the partially unfolding of the insulin structure from pH level of 2.6 and 2 hours of heating at 68 degrees Celsius, using circular dichroism (CD) and fourier transform infrared spectroscopy (FR-IR), the author then used a method of pH level of 2.4 and heating at 60 degrees Celsius which resulted in a brand-new partial fold. (Huus et al., 2005).

1.7.3 Monoclonal Antibody

Monoclonal antibody (mAb) according to the national cancer institute (NIH), is a laboratory-made immune system protein that binds to substrate such as cancer cells. They are made such that they bind to only one substrate. They can be used on their own or as a vehicle to transport radioactive substances, drugs, and toxins directly to the cancer cells. Antibodies are naturally produced by the body assisting the immune system in the recognition of disease-causing agents and immediately marking them for destruction. They can be used to treat diseases such as Crohn's disease, and chronic inflammatory disease (Lundahl et al., 2021). Table 1.4 outlines a list of some approved monoclonal antibody drugs in the market with their therapeutic effects. Monoclonal antibodies (mAbs) are homogenous antibody with constant affinity and specificity requiring sensitive handling and storage from production to the final formulation. They provide new and exciting opportunity for new pharmaceutical development and new approaches to treatment and obstruction of diseases (Jacob et al, 2006).

Table 1.4 List of some approved monoclonal antibody drugs in the market from 2017 to 2021(data extracted from antibody society website and European medicine agency). www.antibodysociety.org/antibody-therapeutics-product-data {accessed 15/08/2022}

Drug	Product	Approval Date	Therapeutic Effects	Manufacturer
Rituximab®	Truxima	17-Feb-17	Inflammations and blood cancers	Celltrion Healthcare Hungary Kft.
Adalimumab®	Amgevita	21-Mar-17	Crohn's disease, moderate to severe ulcerative colitis, rheumatoid arthritis	Amgen Europe B.V.

Adalimumab®	Solymbic	22-Mar-17	Plaque Psoriasis, Psoriatic Arthritis, Rheumatoid Arthritis, Crohn's Disease, Ulcerative Colitis	Amgen Europe B.V.
Rituximab®	Rixathon	15-Jun-17		Sandoz GmbH
Rituximab®	Riximyo	15-Jun-17		Sandoz GmbH
Rituximab®	Ritemvia	13-Jul-17	Non-Hodgkin's Lymphoma,Chronic Lymphocytic Leukaemia, Granulomatosis with Polyangiitis and Microscopic Polyangiitis	Celltrion Healthcare Hungary Kft.
Rituximab®	Rituzena	13-Jul-17		Celltrion Healthcare Hungary Kft.
Rituximab®	Blitzima	13-Jul-17		Celltrion Healthcare Hungary Kft.
Adalimumab®	Imraldi	24-Aug-17		Samsung Bioepis NL B.V.
Trastuzumab®	Ontruzant	15-Nov-17	Breast and Gastric Cancer	Samsung Bioepis NL B.V.
Adalimumab®	Cyltezo	10-Nov-17	Plaque Psoriasis, Psoriatic Arthritis, Rheumatoid Arthritis,	Boehringer Ingelheim International GmbH

			Crohn's Disease, Ulcerative Colitis	
Bevacizumab®	Mvasi	15-Jan-18	Metastatic carcinoma of the colon or rectum, breast and lung cancer.	Amgen Europe B.V.
Trastuzumab ®	Herzuma	08-Feb-18	Early stage of breast cancer	Celltrion Healthcare Hungary Kft.
Trastuzumab®	Kanjinti	16-May-18	Breast and gastric cancer	Amgen Europe B.V., Breda
Infliximab®	Zessly	18-May-18	rheumatoid arthritis, Crohn's disease, ulcerative colitis, psoriasis	Sandoz GmbH
Adalimumab®	Hefiya	26-Jul-18	Severe chronic plaque psoriasis	Sandoz GmbH
Adalimumab®	Halimatoz	26-Jul-18	Active and progressive psoriatic arthritis	Sandoz GmbH
Adalimumab®	Hyrimoz	26-Jul-18		Sandoz GmbH
Trastuzumab®	Trazimera	26-Jul-18	Breast and Gastric Cancer	Pfizer Europe MA EEIG
Adalimumab®	Hulio	16-Sep-18	Plaque Psoriasis, Psoriatic Arthritis, Rheumatoid Arthritis, Crohn's Disease, Ulcerative Colitis	Mylan S.A.S.

Trastuzumab®	Ogivri	12-Dec-18	Breast and Gastric Cancer	Mylan S.A.S
Bevacizumab®	Zirabev	14-Feb-19	Metastatic Carcinoma of the Colon or Rectum	Pfizer Europe MA EEIG
Adalimumab®	Idacio	02-Apr-19	Plaque Psoriasis, Psoriatic Arthritis, Rheumatoid Arthritis, Crohn's Disease, Ulcerative Colitis	Fresenius Kabi Deutschland GmbH
Adalimumab®	Kromeya	02-Apr-19	Rhematoid arthritis	Fresenius Kabi Deutschland GmbH
Netakimab®	Efleira	2019	Plaque psoriasis	Biocad Russia
Prolgolimab®	Forteca	2020	melanoma	Biocad Russia
Levilimab®	Ilsira	2020	Covid-19	Biocad Russia
Olokizumab®	Artlegia	2020	Rhematoid arthritis	R-Pharm Russia
Pabinafusp alfa®	Izacaro	March 2021	Hunter syndrome	JCR Pharmaceuticals
Sotrovimab®	Xevudy	August 2021	Anti SARS-COV-2	Glaxosmithkline plc
Regdanvimab®	Regkirona	September 2021	Anti SARS-COV-2	Cellrion healthcare Hungary Kft

Engineered proteins such as mAbs conjugated with small molecule drugs, bispecific mAbs, multi specific fusion proteins, proteins with optimized pharmacokinetics are currently being developed (Dimitrov, 2012). They are immunotherapy because they can help turn the immune system against the cancer and interact with specific target cells.

The speedy growth of therapeutic mAb research in the pharmaceutical sector is overwhelming, several of the antibodies have undergone clinical trial making a lot of multiple trial products compete for targeted diseases, as there have been several antibody therapeutic drugs targeting antitumor necrosis factor alpha (TNF-alpha) approved by FDA. The evolved antibodies now battle physiological stress within circulatory and immune systems of mammals. They are also seen in millimetre concentrations in vivo. The manufacture of recombinant mAb subjects immunoglobulin to various stress, such as high concentration, pH, heat and mechanical strain. The idea of patients administering the drugs by themselves through subcutaneous delivery route is fast increasing the clinical and commercial needs, hence making the mode of delivery an additional requirement in the manufacture and development of the antibody drugs. It therefore requires the liquid formulation to be of high concentration of more than a 100mg/ml (Dimitrov, 2012). Table 1.5 details formulations and excipients used in various research studies from 2011 to 2022.

The mAb drug final formulation, should be cautiously chosen within an appropriate scale, optimizing the stability and quality of the drug product during manufacturing (Lowe et al., 2011; lahlou et al., 2009; Shire et al., 2004).

Table 1.5: List of antibody formulations and excipients used in various research (2011-2022)

Name of author/ year	**Technique used**	**Mab used**	**Excipient**
Falconer et al., 2011	DSC	IgG1 in CHO cell	Arginine Histidine lysine

Thakkar et al., 2012	DSC, High-resolution ultra-sonic spectroscopy Red- Edge excitation spectroscopy	IgG 0.1mg/mL	NaCl
Manikwar et al., 2013	DSC Size exclusion Performance liquid chromatography	IgG1/ sigma/ 5mg/ml	Sucrose arginine
Neergaard et al., 2014	DSC Dynamic light scattering SEC	IgG1, IgG 4	histidine
Kheddo et al., 2014	Osmostat Light scattering and intrinsic fluorescence SE-HPLC	IgG1	Arginine glutamate
Douglass et al., 2015	Size exclusion chromatography Image capillary isoelectric focusing LC-MS Peptide map mass spectroscopy	IgG1 in CHO cell / 5mg/ml	Sucrose trehalose Hydroxyl propyl B-cyclodextrin polysorbate 80 (
Garidel et al., 2015	High performance size exclusion liquid chromatography (HP-SEC) Hydrophobic interaction chromatography (HIC) Micro flow imaging (MFI)	IgG1 produced by mammalian cell culture in CHO cell line	Arginine Polysorbate 20 Glycine/mannitol Sucrose/mannitol

Startzel et al., 2015	Freeze drying microscopy Modulated differential scanning calorimetry X-ray powder diffraction measurement Karl Fischer moisture determination Size exclusion chromatography	IgG1(humanised) 76.4mg/ml stock solution in 20mM histidine buffer	Sucrose L-arginine L- histidine base Citric acid monohydrate Polysorbate 20
Wang et al., 2015	SEC DSC Dynamic light scattering Fourier transform infrared spectroscopy	IgG1	histidine
Nicoud et al., 2015	UV HPLC SEC	IgG1	Histidine Arginine
Wang et al., 2017	Circular dichroism, Size exclusion chromatography, fluorescence spectroscopy, Differential scanning Calorimetry	IgG	Pluronic 68, F127, Tween 20 and 80, Chaps, Brij35, SDS and TrX-100
Singh et al., 2017	Near UV circular dichroism, 2D NMR, Raman spectroscopy. Size exclusion chromatography, Differential scanning calorimetry and Chemical denaturation melt. Analytical ultracentrifugation	IgG1	Tween 20 and 80

Xu et al., 2019	Dynamic light scattering, Electrophoretic light scattering, Small angle X-ray scattering, DSC, Microcapillary viscometry	IgG	Glucose, sucrose, trehalose and mannitol, Polysorbate 20 and 80
Kuzman et al., 2021	SEC chromatography, Cation exchange chromatography SDS capillary electrophoresis	IgG1, IgG2	Polysorbate 80 Mannitol, sorbitol, sucrose, lysine
Bhojane et al., 2022	Osmolyte effect Accelerated study DSC, Fluorescence spectroscopy, Hemolysis assay	IgG1	Tween 80 Betaine, Sarcosine, Ectoine, Hydroxyectoine
Bluemel et al., 2022	Stability analysis (Long term storage at –80 degrees Flow imaging microscopy, SEC chromatography	IgG1	Sucrose

1.7.3.1 Structure of Antibody

The traditional model of an antibody is a molecule in the shape of a Y. It is made up of four polypeptide subunits, each of which has two heavy chains and two light chains that are identical (Figure 1.9). The fragment antigen-binding (Fab) domains are formed when the N-terminus of each heavy chain associates with one of the light chains to form two antigen-binding domains. These antigen-binding domains are the arms of the "Y" shape and are referred to as the (Fab) fragment antigen-binding domains (Kohler and Milstein, 1975; Sadeghalvad and Rezaei, 2021).

The fragment crystallization (Fc) domain, also known as the "tail" of the "Y" shape, is created when the C-termini of the two heavy chains join with one another to produce a new structure. Both the activation of the complement cascade and the engagement of the antibody with effector cells like macrophages are dependent on the Fc domain. The Fc domain is particularly important in both of these processes. The four polypeptide chains are held together by both covalent and non-covalent connections, specifically disulfide bridges (Kohler and Milstein, 1975).

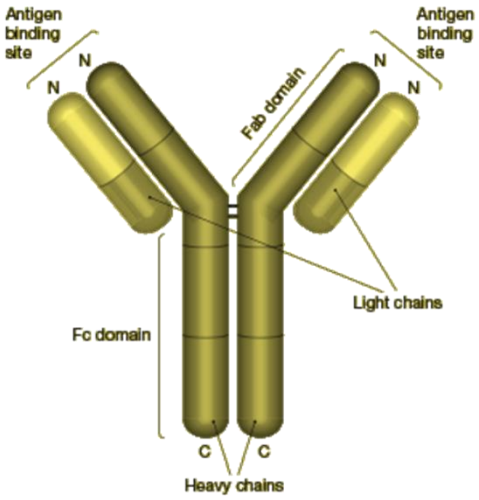


Figure 1.9: Schematic structure of an IgG molecule (Kohler and Milstein, 1975; Sadeghalvad and Rezaei, 2021)

An examination of the sequences of several different light chains have shown two distinct regions: a highly variable N-terminal area (VL) and a consistent C-terminal region (CL). Studies that were conducted in a similar manner on heavy chains discovered that these chains also contain variable and constant regions, denoted as VH and CH, respectively. (Kohler and Milstein, 1975; Sadeghalvad and Rezaei, 2021). IgG heavy chains, on the other hand, have only one variable region (located at the N-terminus), in addition to three constant sections. The variable portions of both chains are found in the Fab domains, and these regions provide the structural basis for the antigen and epitope selectivity of antibodies (Kohler and Milstein, 1975; Wang et al., 2008).

The actual length of light chains is close to 220 amino acids, and their length can be partitioned into two equal parts known as the constant and variable sections. the heavy chains have a length of approximately 440 amino acids and are broken into about four segments of 110 amino acids (Figure 1.10) each. There is one variable section with three constant parts inside a heavy chain. The sequence variability that is seen inside the variable sections is not random, but rather occurs at three brief segments inside each chain that are known as the hyper variable regions. These segments range in length from five to thirty amino acids. As a result of the fact that the residues in the hyper variable areas provide the actual binding site for the antigen, it is common practice to refer to these regions as the complementary determining regions (CDRs) (Kohler and Milstein, 1975; Sadeghalvad and Rezaei, 2021).

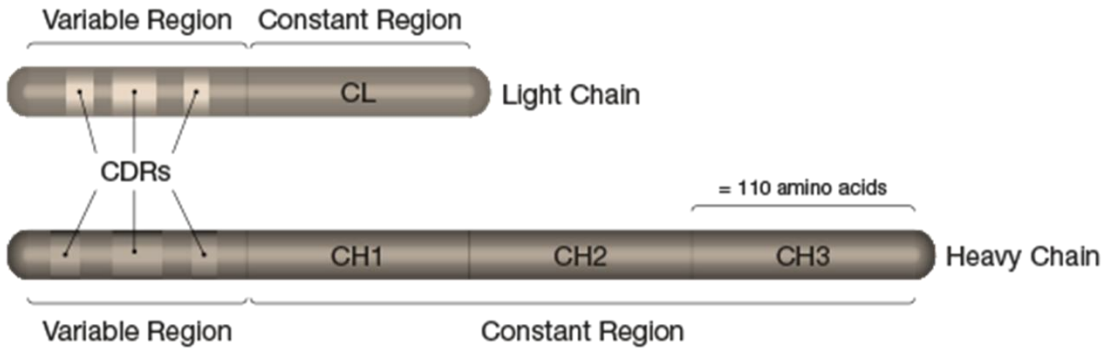


Figure 1.10. Light and heavy chain structure (Kohler and Milstein, 1975)

Antibodies are biological macromolecules that are spontaneously created by the immune system. They are abbreviated as "Abs" the molecular structure is made up of four polypeptide chains, two of which are heavy with a molecular weight of around 50 kilodaltons, and the other two are light with a molecular weight of approximately 25 kilodaltons. The heavy chain is composed of four structural domains, three of which are constant (CH1, CH2, and CH3) and the fourth one is changeable (VH); while the light chain is made up of two structural domains, one of which is constant (CL), and the other is variable (VL) see (Figure 1.10). In the review article that was written by Wang and colleagues, there is a comprehensive explanation of the structure of the antibody (2008). IgM, IgD, IgG, IgE, and IgA are the five classes of antibodies

that can be distinguished from one another according to the sequencing of the constant region of the heavy chain. Following the order of their relative abundance in plasma, IgGs are further subdivided into IgG1, IgG2, IgG3, and IgG4, respectively (Garidel et al., 2020). Additionally, each of these possesses a unique heavy chain, specifically the 1, 2, 3, or 4 depending on the case (Wang et al., 2008).

The earliest application of antibody-based therapy was in the treatment of cancer; while it was first targeted mostly against lymphoma and leukaemia, it was also employed for the treatment of solid tumours like melanoma (Goswami et al., 2013).

1.8 Rationale of this Research

Shelf-life of protein is the major concern in pharmaceutical development. Usually, a formulation-based protein medicine protects its protein from acute damage during processing and shipping. It should permit a storage of about 24 months and above. The major goal for formulation of protein therapeutic drug is to improve storage stability, functionality and robustness of their manufacturing process via an enhanced knowledge of the formulation design. Examples include the excipient type, the concentration of any additive, the container and the closure, effect of the environment like temperature, pressure and pH. Many research studies have looked at different ways to stabilize protein, it has brought about the use of various excipients, adjustment of pH, temperature etc as well as appropriate technique. Spray drying is often used to increase the stability and shelf life of pharmaceutical products and is often preferred (Tian et al., 2007). It has been researched to be a viable alternative formulation strategy in the improvement of long-term storage and stability (Awotwe-otoo, 2013).

Pluronic are known for their excellent biocompatibility and their amphiphilic properties, they are widely used in drug delivery applications, the PPO blocks in pluronic have been observed to carry hydrophobic drugs while the PEO chain has a stabilising effect. Pluronic F-127 has

been used in different types of proteins, very few research had been conducted on insulin stability hence the need to consider it as an excipient in this research study.

Beta cyclodextrin have been reported to have aggregation stabilizers due to their ability to associate with protein, there are also evidence for their role in preventing proteins from being exposed to water and air thereby serving as an aid in preserving the integrity of protein. There are a few research conducted with the use of betacyclodextrin in stabilizing insulin and immunoglobulin in present research hence the need to create a research novelty with these excipients. The rationale for the concentrations applied in this study were based on preliminary information published in previous research study. Most research work for instance on monoclonal antibody have worked on very high concentration between 50mg to 100mg hence the necessity to create a research novelty by looking at a very lower concentration.

1.9 Research Novelty

The original contribution to knowledge was to identify appropriate combinations of excipients and techniques for stability and design formulations in different terms like potency. According to various research, amino acid is said to be the best stabilizing agent, the current challenge is creating or strategizing on the right formulation. Falconer et al, 2011 and others think arginine and glutamate are better stabilizers and some also think amino acids with sugar should be combined. This research intended to investigate a type of surfactant with a combination of sugar and buffer at different formulations concentration in dried state and solution state.

In this case, the sugar and surfactant used (Beta cyclodextrin and pluronic F-127) at a concentration of 1%w/v, 1:1%w/v and 1:5%w/v ratio (protein: excipient; 1%w/v lysozyme + 1%w/v beta cyclodextrin, 1%w/v lysozyme + 1%w/v pluronic F-127, 1%w/v lysozyme + 5%w/v pluronic F-127; 1%w/v Insulin + 1%w/v beta cyclodextrin, 1%w/v Insulin + 1%w/v pluronic F-127 and 1%w/v IgG + 1%w/v beta cyclodextrin) which have not been previously used demonstrated several promising qualities on lysozyme with the drying technique applied.

Electrospray technique in the presence and absence of excipients improved lysozyme stability. In general, beta cyclodextrin used with every model of protein showed a form of possible stability at a low concentration.

Some of the findings observed in this research have been published and presented in various conferences, others will be published in due course.

The formulation derived from this research would be of interest to the pharmaceutical company.

1.10 Research Aims:

- The main aim of the study is to stabilize selected proteins namely lysozyme, insulin and Immunoglobulin IgG in vitro formulations.
- To investigate the influence of drying methods (Spray drying and Electrospray) and excipients (Beta cyclodextrin and Pluronic F-127) on the protein models chosen using various techniques.

1.11 Objectives:

1. Preparation of dried formulations using Spray-drying and Electrospray techniques where the protein drug was prepared in 1:1%w/v ratio with beta cyclodextrin and 1:5%w/v ratio with Pluronic F-127.
    -
    - Studying the thermal stability of proteins in dried and solution forms under several conditions using Differential Scanning Calorimetry and Dynamic Light Scattering techniques
    - Characterization and evaluation of different states of protein and the effect of technique (Spray dry and Electrospray) and excipients with other instruments

namely Ultraviolet-Visible Spectroscopy, High Sensitivity Differential Scanning Calorimetry and Scanning Electron Microscope.

- Investigation of the influence of excipients on proteins in dried and solution forms.
- Assessment of the effect of the presence and absence of beta cyclodextrin on thermal denaturation and aggregation of protein using high-performance liquid chromatography (HPLC).
- Exploring the effect of species and solution conditions on thermal denaturation.

1.12 Structure of the Thesis

Chapter One - introduces therapeutic protein. A background of general protein, formulation development, problems faced with production of protein therapeutic and ways to improve and overcome these challenges.

Chapter Two - describes the research and method in details applied during the research study.

Chapter Three - describes the characterization of lysozyme and the comparison between the drying technique (Spray dry and Electrospray) applied to lysozyme formulations and the effect of excipient (Pluronic F-127 and Beta cyclodextrin) on lysozyme.

Chapter Four - characterizes insulin in dried and solution form using scanning electron microscope, high sensitivity differential scanning calorimetry. It further evaluates the thermal stability of insulin in the presence and absence of beta cyclodextrin and pluronic F-127. In addition, the effects of excipients (beta cyclodextrin and pluronic F-127) and scan rate (10˚C/hr and 60˚C/hr) on insulin is investigated.

Chapter Five - evaluates the thermal stability and aggregation of Monoclonal antibody (Immunoglobulin IgG) by observing the effect of excipients (beta cyclodextrin), source of the

protein on the denaturation temperature. Effect of pH on thermal denaturation and aggregation using high sensitivity differential scanning calorimetry, dynamic light scattering and size exclusion-high performance liquid chromatography as characterization technique.

Chapter Six - Conclusion and future work.

Chapter Seven – References.

Chapter Eight – Appendices.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Protein models

Table 2.1: List of protein models

Protein Model	**Product Information**
Lysozyme (chicken egg white)	lyophilised powder (90%), by Sigma Aldrich UK (L6876-25G), Pcode: 1002188661
Insulin human	Purchased from Sigma Aldrich. UK Molecular weight 5807.57g/mole, Pcode: 1002767117, CAS: 11061-68-0, powder form
Lantus vial insulin	Purchased from AAH pharmaceuticals UK Pcode:0501301105068. Sanofi Aventis Germany
Bovine Serum IgG	Sigma Aldrich (15506-100mg) lyophilized powder. Lot # SLCD7881; Pc 1003050619 USA
Immunoglobulin (IgG) human serum	Sigma Aldrich (56834-25mg; 14506-100mg) lyophilized powder. Lot #SLCF7655; Pc 1003076078 USA
Antichop antibody (mouse monoclonal antibody)	Produced and purchased from Johns Laboratory London, UK. Model ST J97774

2.1.2 Chemical and Reagent

Table 2.2: List of chemicals and reagents

Chemicals/reagents	**Product information**
Micrococcus lysodeikticus	lyophilised cells, Sigma Aldrich UK (M3770-5G) Lot#SLBM7420V, Pcode: 1002295445, ATCC No 4698
Sodium phosphate dibasic (HNO_20_4)	Purchased from Sigma Aldrich UK. molecular weight 114.96, CAS: 7558-79-4, Pcode: 101310493 lot-BCBK4050V
Potassium Phosphate Monobasic **(**KH_2PO_4**)**	BP 362-1purchased form Fisher scientific. UK. Molecular weight 136.09, CAS:7778-77-0, Lot :090620
Disodium hydrogen orthophosphate anhydrous	Molecular weight:141.96, EINECS: 231-448-7, Code: S/4480/60, Batch 0876220. Fisher scientific UK.
Sodium chloride	Mw 58.44 S/3160 Fisons Scientific company. UK.
Potassium Chloride (KCl)	MW 74.55g/ml CAS no: 7447-40-7, Lot: K51293909938 Merck Germany.
Tween 80	Fisher Scientific company UK.
Ethanol, C_2H_6O	Lot BCBT6013 CAS:64-17-5, MW:46.07 g/mol, code:02851 Sigma Aldrich UK.
Nitric acid	Fisher Scientific company UK.
Hydrochloric acid (HCl)	Lot: SZBF1790V Code: 258148 CAS 764-01-0 MW 36.46g/mol Sigma Aldrich UK.

Sodium hydroxide	Fisher Scientific company UK
Trifluoroacetic acid	Fisher scientific UK, Lot: 1490760
Acetonitrile	Lot: 2049762, Code: A/0626/17 MW 41.05, CAS 75-05-8 Fisher scientific UK
Sodium citrate	$C_6H_5NA_3O_7.2H_2O$, S-4641, Lot 101H0285, MW 294.1 Sigma UK
Sodium lauryl sulphate (SLS)	Fisher Scientific company UK
Deionized water/ mili-Q water	University of Sunderland laboratory UK

2.1.3 Instruments used for Research.

Table 2.3: Instrumentations

Instruments/Apparatus	**Model and Manufacturer**
Spray dryer	Mini- Buchi B –290, Switzerland
Electrospray apparatus	Harvard Apparatus, Pump 11 Elite, USA
High-power voltage supply	Glassman High Voltage Supply, UK
Microcal- VP Differential Scanning Calorimetry	Malvern instrument UK
UV-Vis spectrophotometer	M501 Single Beam Camspec by Biochrom, UK
Scanning Electron Microscope	Hitachi S-3000N Hitachi limited Japan
Thermogravimetry Analyser (TGA)	Mettler Toledo UK
Turbidometer	Hach Ratio/Xr model
Differential scanning calorimetry	Q1000M TA instrument, England.
pH meter	Hanna. USA

Zetasizer	Malvern zeta sizer nano ZSP UK
Oven	Memmert UNE 400 Germany
HPLC	Agilent 1290 Infinity LC system Agilent USA
ACQUITY UPLC Protein BEH SEC Column 200 A.	WATERS UK S/N 186005793
MAbPac RPTM Column	Thermofisher Scientific UK. S/N 001066
Weighing balance	Precisa 125A, Switzerland
Water bath	Hilsonic UK

2.2 Methods

2.2.0 Protein Preparation Techniques

2.2.1 Solubility of Protein

The quantity of protein in a sample that is able to dissolve in a solution is referred to as the sample's solubility. The percentage of nitrogen in a protein product that is soluble when subjected to particular conditions is what is meant to be understood as the solubility of protein. (Zayas, 1997) Both the protein and the additive have the potential to be totally soluble in water, somewhat soluble, or totally insoluble. The medicine samples' solubility is of the utmost importance.

2.2.1.1 Solubility of Lysozyme sample

Lysozyme sample was dissolved in water before spray drying process and later dissolved with PBS buffer after spraying for analysis**.**

2.2.1.2 Solubility of Insulin Sample

The solubility of insulin is dependent on several factors which include the temperature and concentration of salts and metal ion and the nature of solvents, pH. Insulin has an isoelectric point of 5.3 and it is almost insoluble to dissolve in neutral conditions, however, it can be easily dissolved on diluted acidic or alkaline solution. For this research, different concentrations of hydrochloric acid at different molarity were used and finally arrived at 0.1M HCl to dissolve the insulin power at a pH of 1.2.

2.2.2 Drying of the Proteins

Drying of protein is the removal of water from the liquid protein turning them into solid powder form.

2.2.2.1 Preparation of Spray-dry lysozyme

The lysozyme samples were prepared for spraying by measuring or weighing 1% w/v protein with and without 1%w/v excipients solution hence 1g of protein and 1g of excipients (beta cyclodextrin or pluronic) using (Precisa 125A) weighing balance and dissolved in 100ml of distilled water (Appendices ii). An example, shown in Figure 2.3, a spray dryer model called a Mini Buchi B-290 being used to spray the aqueous protein solution. A silicone tube was used to send the feed solution concentrations prepared to the chamber for drying through a peristaltic feed pump flowing at a 20 millilitres per minute (ml/min) rate, where they were dried at 130.3 degrees Celsius temperature inlet and 73 degrees Celsius temperature outlet. In

order to reduce levels of stress and protein breakdown, there was circulation of cooling water through the jacket surrounding the nozzle. The spray-dry protein powdered samples were carefully collected from the particle collector and later stored in a vial and put a desiccator and stored in the fridge at 3-4°C

2.2.2.2 Preparation of Spray-dry insulin

Various concentrations of 1% insulin solution (1g of insulin in 100ml of distilled water) with the addition of some excipients (betacyclodextrin, pluronic) was prepared and spray dried using a Mini Buchi Spray Dryer B-290 model of spray drier. The prepared concentrations of feed solution were passed from a pipe through the atomising nozzle of 0.5mmdiameter to the drying chamber via a peristaltic feed pump at a flow rate of 20ml/min, dried with an inlet temperature of 110-130±3 ^{o}C, outlet temperature of 73±^{o}C. Cooling was circulated through the jacket around the nozzle for protein degradation minimisation. Spraydried powder was collected in the cyclone separator, stored in a vial and stored in the fridge.

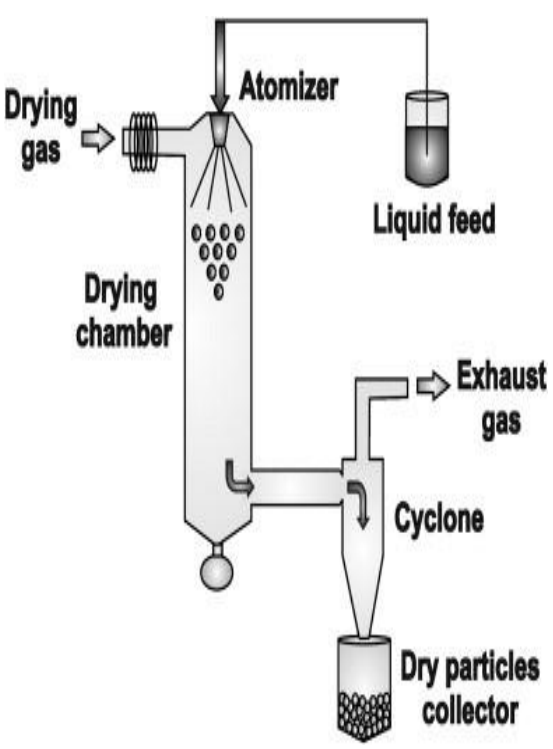


Figure 2.2: Illustration showing a spray drying process. (Sosnik, & Seremeta, 2015) and Mini Buchi-290 Spray Dryer. (www.sisc.com accessed May 3, 2017)

2.2.2.3 Preparation of Electro sprayed Lysozyme

1% w/v protein solution with and without excipients was prepared by dissolved in 80:20%v/v ethanol and water hence 1g of lysozyme and 1g of excipients (beta cyclodextrin or pluronic) in 80ml of ethanol and 20ml of water, The solution was homogenised using magnetic stirring, and then it was placed into a 5 ml syringe that was coupled to a programmable syringe infusion pump that was supplied by (Harvard Apparatus, Pump 11 Elite, USA) and attached to a high-power voltage supply (Glassman High Voltage Supply, UK). Figure 2.3 gives an illustrative view of the process. The protein solution was passed through a silicone tube connected to the syringe and the axial nozzle compartment and pressurised slightly for an achievable stable spraying. With a flow rate of 15 microliters per minute and a needle diameter of 0.3 millimetres, a positive high voltage was employed to maintain the voltage range of 8-18 kilovolts. A microscopic collecting slide was placed below the needle tip on a collector plate (Figure 2.4) and is operated by a stepping motor with a distance between 13cm to 15cm during spraying to obtain the atomised particles.

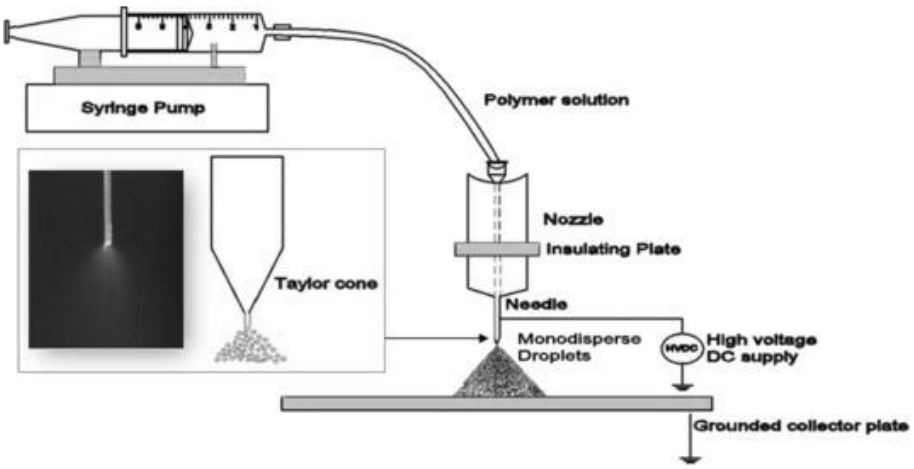


Figure 2.3: Showing an electrospray set up (Bhushani and Anandharamakrishnan, 2014)

2.2.3 Percentage Yield

Percentage yield is an actual yield in the mass of a product usually obtained from the formulation. It is mostly less than the theoretical yield as a result of practical loses during the experiment such as filtering, pouring and spraying. The percentage yield is calculated using the equation below

Percentage yield = actual yield (product mass obtained) /theoretical yield * 100-------I

The actual yield of SD and electro sprayed lysozyme sample obtained was divided by the initial weight before spraying and multiplied by 100

2.2.3.1 Percentage yield analysis

For the percentage yield measurement, the exact weight of protein sample was weighed and recorded before dissolving the native protein in a solution in preparation for spraying and the dried protein sample obtained after spraying was collected in a clean vial, weighed with a Precisa 12A weighing balances and recorded before storage. The percentage yield was calculated with the equation I stated above.

2.2.4 Biological Activity Analysis

The biological activity was obtained with an established enzymatic potency assay. The activity of lysozyme was assayed by hydrolysing a bacterial solution. It was measured with the basic and kinetic mode using the UV-Vis spectrophotometry (Camspec, M501). The protein (lysozyme) lyses various microorganisms through the hydrolysis of the β (1-4) glycosidic bonds that comprises their cell walls and exhibiting bacteriostatic properties. The activity depends on the ability of lysozyme to lyse a suspension of the bacteria of a known concentration. During this process, the decrease in turbidity of the prepared suspension is measured.

2.2.4.1 Biological Activity Analysis of lysozyme

The bacterial solution was prepared by adding 20mg of Micrococcus Lysodeikticus in phosphate buffer solution containing 90 millilitres of 0.0067M solution with a pH of 6.24 and ten millilitres of 1% sodium chloride.

A buffer containing 25 millilitres of solution containing 0.01gram of the produced formulation and 0.01 percent (w/v) of each sample. After adding 0.5 millilitre of each produced protein to 2.5 millilitres of bacterial suspension, the biological activity was measured by observing a decline in absorbance using an M501 single beam UV-Vis spectrophotometer set to 450 nanometres. This method was used to analyse the protein. The duration of the activity was measured in minutes as (0, 1, 2, 3). The Shugar 1952 equation was utilised in order to arrive at the conclusion concerning the outcome of the activity. A microsoft excel spreadsheet software was used for the analysis (see appendices v)

Activity (units/mg) =$\Delta 450_{nm/min}$/ 0.001 x mg enzyme present in the mixture. ----------II

2.2.4.2 Biological Activity Analysis of lysozyme using KINETICS mode

The bacterial solution was prepared with (20mg%) of Micrococcus lysodeikticus in phosphate buffer. A concentration of 20g in 90ml of 0.0067M, at a pH of 6.24 and 10ml of 1% NaCl was prepared. 2mg of each sample of lysozyme was measured twice in two different beakers of 25ml buffer; the solutions were kept in the water bath for 15mins at 25°C. Also, the bacterial solution was kept at the same temperature. The biological activity was measured with a UV-Vis spectrophotometer (Camspec, M501) and set to a kinetics mode with the following settings.

Total time: 180s, Time interval: 1s, Delay time: 0s. Wavelength 450nm. The equipment was blanked with 2ml of bacterial suspension and 1ml of phosphate buffer at a pH. of 6.24. The biological activity was measured using 2ml of the bacterial solution and 1ml of each lysozyme

twice and each of the protein sample reading was read three times. The biological activity was then calculated with the formula below.

Biological Activity= IU/ (0.001 * m) ----------III

Where (m) is the weight of lysozyme in milligram that is present in the cuvette, reaction mixture.

2.2.5 Storage Stability Studies

The dried powders were stored in a glass vial, put in desiccator, and stored at a temperature 4°C. The samples were intended to be analysed at 6 months following room temperature conditions however, ended up being analysed after 8 months using the high sensitivity DSC.

2.2.6 Thermal Analysis of Protein

Thermal stability study was carried out using:

- Microcalorimetry VP-DSC (high sensitivity DSC) manufactured by Malvern instrument UK for liquid protein sample solution.
- Differential Scanning Calorimetry for solid protein samples.

2.2.6.1 High Sensitivity Differential Scanning Calorimetry (HSDSC)

High Sensitivity DSC also known as Microcal VP- Differential scanning calorimetry was used in this study to determine the thermal behaviour and folding reversibility of lysozyme.

Many literatures reports have cited the value of HSDSC for the determination of thermodynamic parameter which clearly describes the unfolded and folded state of protein. It is used to measure the thermal transition reversibility that is no less important than $T_{m.}$ and ΔH. Furthermore, a protein transition is considered reversible if the molecules renature upon cooling after heating. HSDSC provides details of thermal stability and instability of solutions

of different formulation including the formation of soluble and insoluble aggregate. Additionally, it is used in the assessment of thermal stability of lysozyme solution after storage at stressed conditions such as a temperature of 60 degrees (Elkordy et al., 2012).

Figure 2.4 Showing a picture of the high sensitivity differential scanning calorimetry system.

2.2.6.1.1 Principles of operations.

The (Tm) transition midpoint is defined as the temperature at which half of the protein is in its native conformation and the other half is denatured. A higher Tm value indicates a more stable molecule. ΔCp which is associated with the unfolding process of proteins, occurs as a result of hydration changes in the side chain exposed to solvent in the denatured manner and is however buried its native state. The total integrated zone below the transition peak is denoted by calorimetric enthalpy (ΔH). It depicts the total heat energy uptake by the sample following appropriate baseline correction, which influences the transition. Van't Hoff enthalpy ($\Delta H_V H$) is an experimental model-independent measurement of transitional enthalpy (Gill at al., 2010).

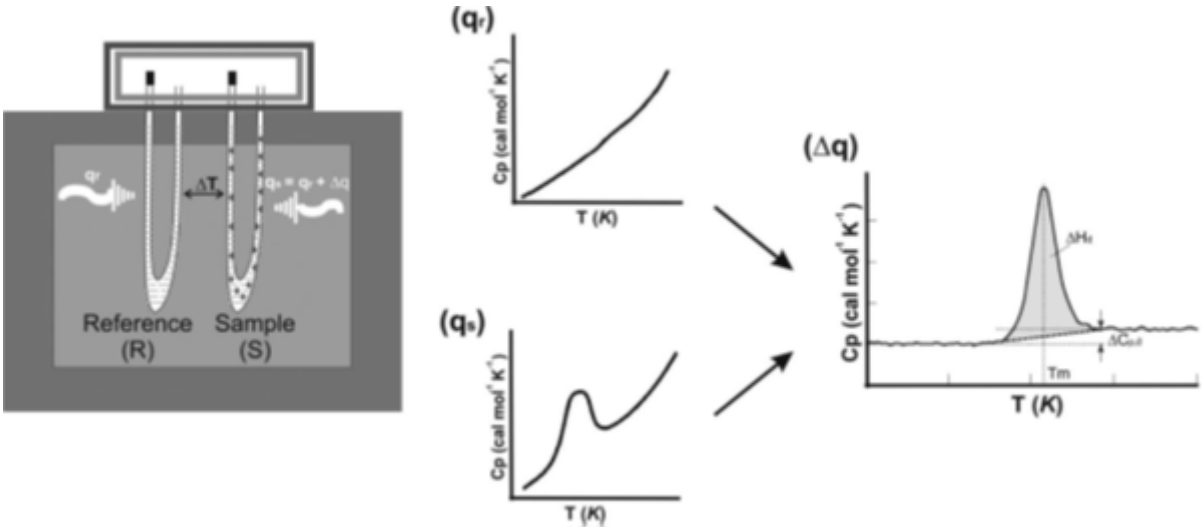


Figure 2.5: illustration of a DSC process (Gill et al., 2010)

2.2.6.1.2 Maximising the DSC Performance

The DSC is very capable of detecting various heat capacity changes of several sample solution.

There were routine procedures carried out manually which include:

- Sample preparation
- Determining the concentration
- Degassing the sample and reference solution
- Cleaning and rinsing the cells
- Loading the solution into the cells

When the sample running procedure on the DSC, appropriate measures were taken for accurate result.

The best results were obtained when appropriate techniques were mastered because it is very difficult to get a reading if sample solutions are extremely diluted however, several points were emphasized.

It has been stated by Malvern that sometimes biological solute may precipitate slightly during degassing procedure or proteins may not dissolve completely. In this case, visual inspection

of the solution was carried out under appropriate lighting conditions which revealed a clear solution or small particles suspended in the sample solution. However, any particle observed, was removed with a disposable syringe filter with low binding capacity for biological solute. Precipitation during degassing was completely avoided by using a short degassing time of about 3 minutes and stirring of the samples were done at room temperature or avoid degassing by preparing solutions from degassed buffer solution. Furthermore, in carrying out different related experiment, it was important to get the best comparative result, each sample solution was treated the same. The DSC instrument was further calibrated with water for maximum performance. Figure 2.7 and 2.8 shows the DSC temperature calibration and report.

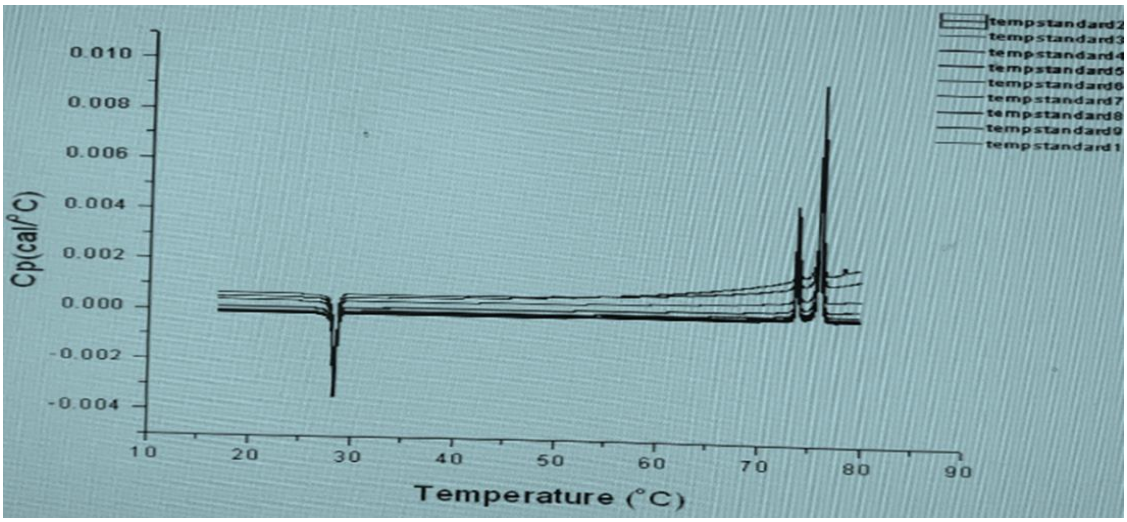


Figure 2.6 DSC temperature calibration

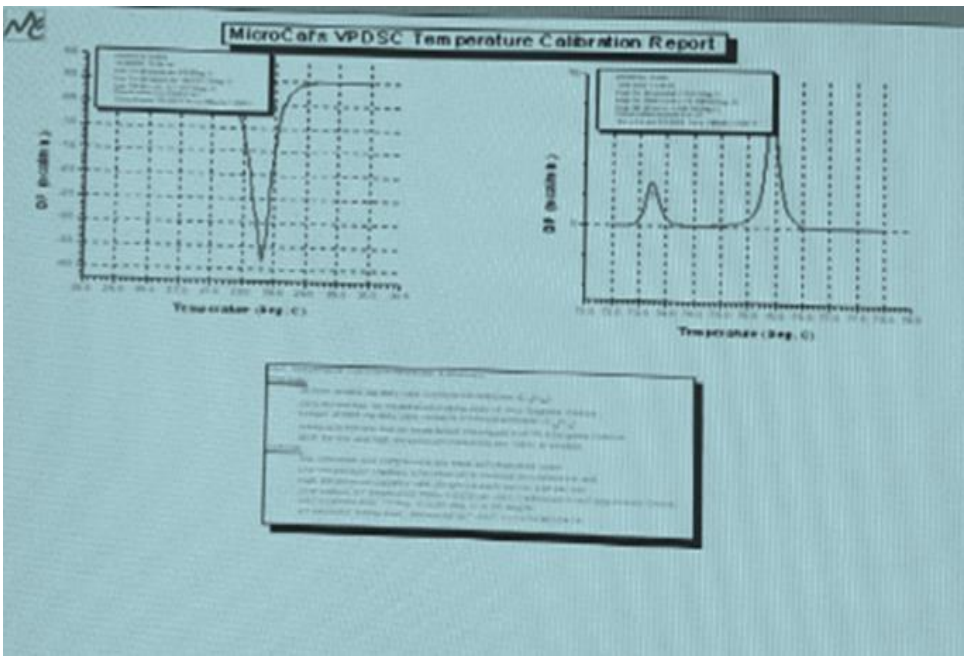


Figure 2.7 Temperature calibration report

2.2.6.1.3 Lysozyme Analysis using HSDSC.

The folding reversibility and thermal behaviour of lysozyme solution were studied using HSDSC. 5mg of lysozyme dissolved in 0.1M sodium phosphate buffer with a pH of 6.24) and the reference solution (a 0.1M phosphate buffer with a pH of 6.24) were degassed before being injected into the cells with a syringe. Both the sample and the reference cells were equally filled so that the volumes could be maintained at the same level, and the exact same amount of lysozyme was utilised in each run. Both the sample and the reference were heated using VP-Microcal DSC, Malvern United States, between 20 and 90 degrees Celsius at a scan rate of 60 degree Celsius per hour. The reversibility of the denatured protein's folding was determined by cycling the temperature via a series of three scans: an up scan that went from 20 to 90 degrees Celsius, a down scan that went from 90 to 20 degrees Celsius, and another up scan that went from 20 to 90 degrees Celsius. These involved two or more scans carried out at the stated temperatures. In addition, before measuring each sample, a baseline was established by loading both cells (sample and reference cells) with the reference (buffer-buffer); the

baseline was then subtracted from the thermal data of the protein using the Origin DSC analysis software, which was used for sample analysis. The sample was analysed by normalizing the concentration and excesses in heat capacity. The calorimetric enthalpy changes which is the area under the peak (ΔH) and midpoint of the transition peak (T_m) was calculated.

2.2.6.1.4 Sample preparation of insulin solution for Insulin Analysis using HSDSC

The Insulin samples were prepared using 0.1M HCl buffer and adjusted to a pH of 1.2 using sodium hydroxide and hydrochloric acid and 7mM phosphate buffer at a pH of 7.6. Fresh stock sample of insulin was prepared at 100mg/10ml (1% w/v) to a concentration of 1.7mM. The prepared sample was degassed and injected into the HSDSC at a starting temperature of 25 to 95°C and scanned at two different rates of (10 and 60°C/hr) with the help of VP-DSC differential scanning microcalorimeter. All insulin scans were run with 0.1M HCl as reference. Before the concentrations were normalised, a reference scan (buffer-buffer) was subtracted from each scan. For the purpose of the data analysis, origin DSC analysis software version 7.0 was utilised.

For independent non-two-state transitions, curve fitting was carried out using equations IV and V

$$\boldsymbol{C_P(T) = \frac{K(T)\Delta H_{vH}\Delta H}{[1+K(T)]^2 RT^2}} \text{----------IV}$$

$$\boldsymbol{K(T)} = \exp\left[\frac{-\Delta H_{vH}}{RT}\left(1 - \frac{T}{T_m}\right)\right] \text{----------V}$$

where C_p is the heat capacity, T the temperature, K the equilibrium constant of unfolding, ΔH the calorimetric enthalpy, ΔH_{vH} the van't Hoff enthalpy, R the gas constant, and T_m the transition midpoint.

The thermodynamic functions applied by DSC fit are described by Grek et al., 2001.

2.2.6.2 Differential Scanning Calorimetry for Solid (lysozyme powder)

An English-manufactured DSC Q1000 UK thermal analysis device was used to examine a solid sample containing 2-3 mg of lysozyme preparations. Each mixture was then placed into the cells after being sealed in an aluminium DSC pan with covers and also a reference consisting of an empty pan with a cover, a flow rate of nitrogen at 50 ml per minute, equilibrated to a temperature of 10.00 degrees Celsius, ramped 10°C and worked up to 300.00°C.

2.2.7 Moisture Content Analysis using Thermogravimetric Analysis (TGA)

The moisture content of protein sample is determined with thermogravimetric analysis (TGA). The change in weight of a substance or a material was measured by a thermogravimetric analyzer either as a function of increasing temperature or isothermally as a function of time in an air, helium, nitrogen, or other gas atmosphere. They are used in the analysis of polymer, glasses, ceramics and other organic materials for composition and purity analysis as well as moisture content absorbed by samples. The thermogravimetric analysis, or TGA, is a technique that analyses the change in mass of a sample when the sample is heated, cooled, or maintained at a constant temperature. The temperature range of the equipment is between 25 and 900°C routinely with a maximum temperature of 1000°C for some instrument. The instrument consists of a furnace and a highly sensitive balance. The furnace has a heating rate where the compound or sample decomposition depends on the heating rate. When there is a high heating rate, the decomposition temperature is likewise high. A heating rate of 3.5°C per minute is recommended for reliable and reproducible analysis.

2.2.7.1 Lysozyme Sample Preparation

About 2-4mg of lysozyme powder was weighed in an alumina crucible with the TGA Mettler Toledo UK instrument Figure 2.9 and heated to a temperature of 350°C at 10°C/min under continuous nitrogen flow set on the instrument.

2.2.7.2 Insulin Sample Preparation

About 2-3mg of insulin powder was weighed in an alumina crucible in a Malvern Mettler Toledo instrument and heated to a temperature of 50- 350°C at 10°C/min under continuous nitrogen flow set on the instrument.

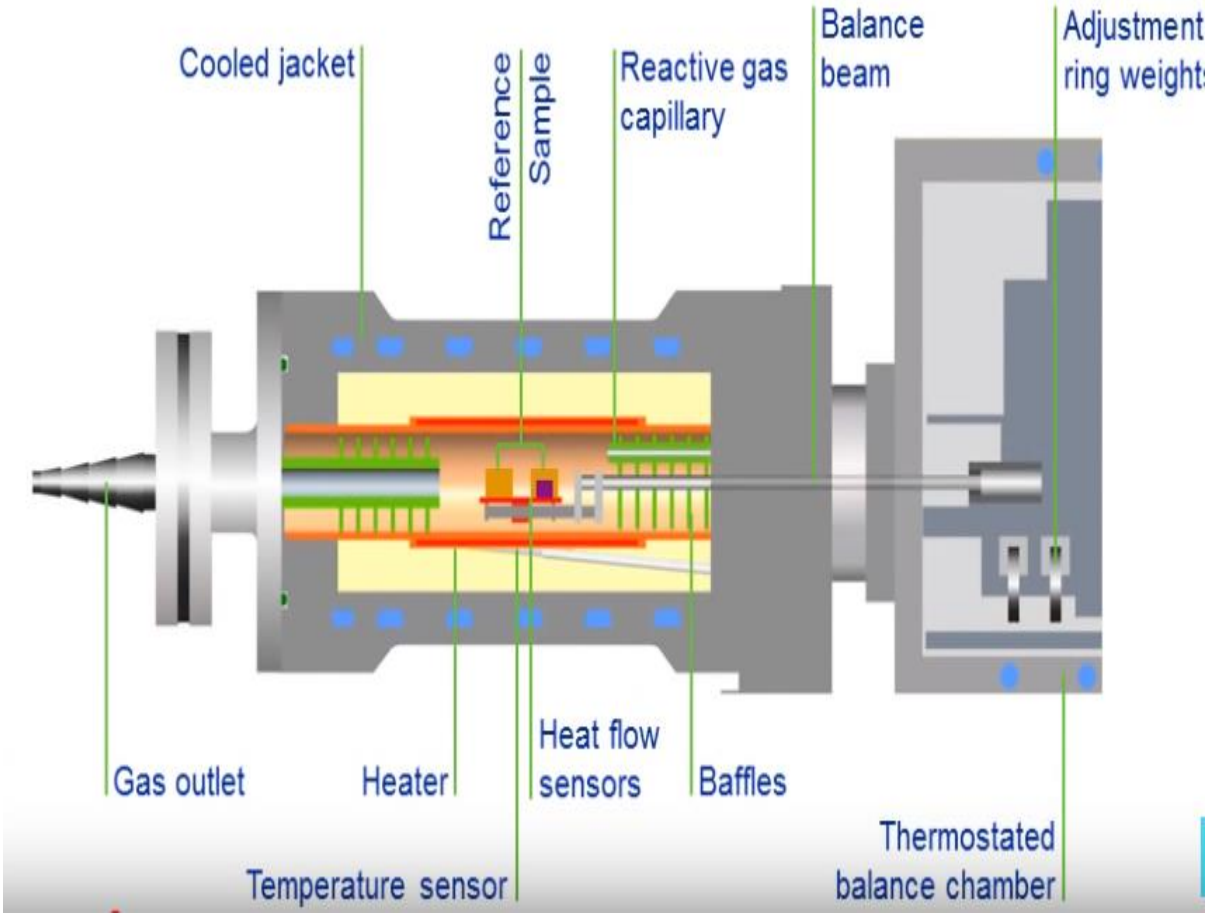


Figure 2.8: illustration of a TGA instrument (Mettler, 2018)

2.2.8 Morphology of Protein Sample

One of the instruments that best describe and analyse protein samples morphology is called Scanning electron microscopy.

2.2.8.1 Scanning Electron Microscopy

The scanning electron microscope is a type of electron microscope that generates a range of signals at the surface of a solid particle by using a high-energy electron focus beam. The signals that are obtained from the interaction between the electron and the sample reveal information about the sample, such as its exterior morphology (texture), chemical composition, crystalline structure, and the orientation of the material that constitutes the sample. In a nutshell, it gives a detailed information on the surface information by tracing a sample in a rectangular pattern. SEM has a variety of application in the scientific industry related field. It is beneficial for solid material characterization like the morphology and composition information. It can also give microstructure details, analyse and detect the surface fracture, contaminations, show chemical compositions of samples, provide qualitative and chemically analyse and identify crystalline structures. It is an essential tool in research due to its practical industrial and technological applications.

2.2.8.2 Scanning Electron Microscopy Analysis

Using a scanning electron microscope, the morphology of the particles was seen. In order to conduct an analysis using a scanning electron microscope (SEM), the previously prepared powdered sample was smeared on a sample holder made of double-sided carbon tape. A significant amount of powder was removed, and in its place, a thin coating of particles was left on the surface of the tape. Using a device called a sputter coater and an electrical potential of 2.0 kV at 25 mA for the full duration of the coating process, gold was sputtered onto the

samples. SEM micrographs were taken with the Hitachi S-3000N utilising the in-built image capturing software.

2.2.9 Particle Size Distribution Analysis

Particle size distribution was used in the determination of the size and morphology of protein sample because it is a scientific way to detect aggregation and degradation of protein. Dynamic light scattering was the chosen technique.

2.2.9.1 Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) is a popular method for determining the size of particles. The sizes are based on the random variations in measurement of the intensity of light scattered from a solution or suspension used to model it. DLS is an acronym for "dynamic light scattering" (Horiba online, 2017). Photon correlation spectroscopy and quasi-elastic light scattering are two more names for this technique. It is a sensitive method that can identify even minute levels of aggregates present in preparations and degradation products. (Bock et al., 2012).

During the process of pharmaceutical development, one of the most important aspects of analytical characterization is size study of peptides and proteins in solution or suspension. Separation-based approaches, in conjunction with a variety of other techniques, such as ultracentrifugation, high-performance size exclusion chromatography (HP-SEC), and asymmetrical flow field fractionation, can be utilised to acquire information regarding particle size (Hawe et al., 2012). DLS is used in the analysis of various protein particle sizes, it can also be used to investigate complicated fluids such as solutions that are highly concentrated. Since proteins have a fairly regular composition and fold into relatively compact shapes, the hydrodynamic size of proteins has a very predictable relationship with their molecular weight.

DLS is a characteristic technique which takes only one minute for measurement while ascertaining the size of biomolecules in solution. It may be used to differentiate between aggregated and homogenous monodispersed samples. The size and intensity during measurement at different temperature provides information about denaturation and melting point which is related to the thermal stability or integrity of a material (Nobbmann et al., 2007) also due to Brownian motion, there is a fluctuation of particles with time. DLS takes measurement of these fluctuations across a very short time interval to produce a single correlation curve that is then deconvoluted using algorithms to obtain information on size distribution.

It measures at a short time scale, the fluctuation of the intensity of scattered light which is used to measure diffusion coefficient that are then used to calculate the Stokes radius or hydrodynamic radius of macromolecules in the size ranging 1 to 1000nm, the molecular weight can be projected from the measured hydrodynamic radius R_H with the help of the molecular weight versus hydrodynamic radius calibration curves developed from standards of known MW and sizes. Hydrodynamic radii are influenced by solvation and the shape of the particles.

The advantages of DLS are the ability of the sample to be analysed with the addition of buffer, without dilution or other manoeuvres required with other techniques (Banga 2015). DLS have been used to investigate aggregation behaviour in several types of proteins such as lysozyme, antibodies solutions. Depending on several factors, the R_H varies. Examples of approximate values are 3.6nm for BSA, 6nm for IgG, insulin may be 1.3-2.6 nm range as the molecules change from monomeric to hexameric structure as a function of a pH change from 2.0 to 7.0 and 1.9nm for lysozyme. Monoclonal antibodies are generally around 12-14nm. Majority of proteins in monomeric form do not exceed 20nm (Das, 2012) In the DLS study, fluctuations in the intensity of light scattering in the protein solution over a time scale from approx. 0.1 µs to approx. 0.1s are due to Brownian motion of the molecule.

2.2.9.2 Mechanism for Dynamic Light Scattering

The small particles experience Brownian motion which is the random movement of particles. This random movement was modelled by Stokes Einstein equation (VI) below and is often used for the analysis of particle size. Basically, this calculation is usually handled by the machine.

$$D_h = \frac{k_B T}{3\pi n D_t} \text{ ----------VI}$$

Where D_h is the hydrodynamic diameter of the particle

K_B is the Boltmann's constant (1.38x 10^{-23} JK^{-1})

T is thermodynamic temperature

π is 3.1416

η is dynamic viscosity

D_t Is the translational diffusion coefficient.

The sample inside the cell is illuminated by the light coming from the laser light source. The dispersed light signal is gathered by one of two detectors, depending on the scattering angle, which can be either 90 degrees (a straight angle) or 173 (a back angle). Because of the availability of both detectors, there is increased leeway in selecting the appropriate measurement conditions. There are several different liquids that are capable of dispersing particles. For understanding the measurement findings, all that is required to be known is the refractive index of the liquid as well as its viscosity (Horiba scientific).

2.2.9.3 Lysozyme Particle Size.

The particle size distribution of the powder was determined by dynamic-light scattering using the Malvern zeta sizer nano ZSP UK. The powders/solutions were dispersed in phosphate buffer, ethanol and tween 80 which was selected to achieve suitable de-agglomeration using a square plastic cuvette from a consumable pack. The cuvette was filled with about 1.0ml-1.5ml of the supplied syringe, the cuvette was tilted to allow the sample fill slowly to avoid bubbles also, the sample depth was between 10 and 15mm, this was crosschecked by placing the cuvette against the diaphram on the inside of the lid of the instrument. Care was taken in holding the cuvette at the top to avoid grease, dust stains or fingerprint stains on the lower area as it may cause the test to fail. The average particle size was measured in three replicates for each sample. The sample cells were thoroughly cleaned especially between different types of samples to avoid cross-contamination which may seriously affect the result. PDI and peak values were derived from the diffraction data using the in-built software (Zeta sizer software 7.11) for each sample. The PDI shows the particle size distribution.

2.2.9.4 Insulin Particle Size

The particle size distribution of the insulin powders was determined by dynamic light scattering using the Malvern zeta sizer nano ZSP UK. 1mg/ml of insulin formulations were dispersed in HCl and PBS which was selected to achieve suitable de-agglomeration. The average particle size was measured in three replicates for each sample. PDI and peak values were derived from the diffraction data using the in-built software for each sample.

2.2.9.5 Monoclonal Antibody Particle Size

The particle size distribution of Immunoglobulin was analysed using size and polydispersity index (PDI) with a technique known as dynamic light scattering (DLS) a Zeta sizer Nano ZSP

analyser instrument Cambridge UK. The monoclonal antibody formulations of 1mg/ml, 2.4mg/ml dissolved in 10mM, 140mM NaCl at a pH of 5.5 and 7.4. The concentration was transferred to the disposable cuvette and placed in the zeta sizer.

2.2.10 Size Exclusion - High Performance Liquid Chromatography

This is an essential technique for analytical and validation characterization of monoclonal antibody and quality control during the development of drug. It guarantees the safe, consistency and stable properties that meet regulatory standards. It is a method used for molecular weight distribution characterization of samples and frequently used in the analysis of proteins to assess aggregate ratio and protein purification. It causes molecules to be separated depending on their molecular sizes, where firstly, the molecules that are bigger are eluted from the columns. The columns can be silica-based or polymer, with the more recent employing a covalently modified diol hydrophilic layer that minimizes non-specific binding of proteins to the underlying silica. To have a true size-based separation, there should be avoidance between the sample and packaging materials. Protein aggregation is easily separated by monomers by size exclusion chromatography, as it is not dependent on any type of interaction. Similar methods can be used to analyse divergent types of mAb and the latest sensational topic is the acceleration of SEC. The reduction in the time taken to analyse per sample is a requirement for maintaining the level of production which would lead to improved Chromatographic mode. However, an impediment for the total downstream process most times considered is Analysis. In 1982, when the earliest recombinant insulin was introduced, almost thirty years after the analysis of protein using SEC was first reported, the most extensively used approach for the regular examination of the bonding or clustering of Biotherapeutic Peptides and Proteins is SEC. The development of the production processes and formulations for these Biotherapeutics has been guided by the extensive utilisation of SEC.

The simplicity, reproducibility, sensitivity, and speed of this approach have led to widespread acceptance for these analyses. SEC's capabilities have recently been strengthened using smaller particle columns (less than 2 microns) on low dispersion UHPLC apparatus, which has produced remarkable gains in resolution, sensitivity, and throughput (Diederich et al., 2011).

To build robust, accurate, and exact methodologies, a complete understanding of SEC principles and practises is required. In theory, the separations of SEC are exclusively reliant upon the size of protein or peptide in solution, but the protein peak, recovery, retention, and shape could have an undesired effect from interactions between the column packing and the large molecules. This should be avoided using technique optimization. In addition, in the sample, the SEC technique development should include a suitable chromatographic recovery assessment of the medicinal drug as well as any aggregate forms (Hong et al., 2012).

The analysis of protein and the aggregation of peptide will be for a long time to come, a significant qualitative feature, because since 2007, the continual entry into the clinic, of innovative peptide-based biotherapeutics and protein has been carried out (Reichert, 2011).

SEC, an almost 50-year-old technology that has evolved to meet ever-increasing demands for correctness and sample output, is now the dominant method for assessing the aggregate levels of protein. Still, SEC suitability for such purpose must be proven for a specific biopharmaceutical sample always (Hong et al., 2012).

ACQUITY BEC column appeared the best selection of column among the evaluated columns for total mAb aggregate quantification, since it allows for a more than two-fold increase in throughput when compared to the TSK gel column (assay time comparison at a resolution. Furthermore, because the ACQUITY column has a relatively modest effect of flow rate on separation, assay throughput was raised without considerably affecting resolution. Lower buffer consumption and sample volume are further advantages of the ACQUITY column (Diederich et al., 2011).

2.2.10.1 Basic Instrumentation Considerations and Method for Optimisation.

Proteins tend to bind to the positively charged areas of stationary phases. Protein adsorption, asymmetry variations (or retention time peak tailing variations), and the three-dimensional structure changes can all come from ionic interactions with other molecules. Nonideal interactions have been mitigated using chromatographic stationary and mobile phases, as previously indicated (Tantipolphan et al., 2010). It is possible to adjust SEC separations such as column length, flow rate, mass load, and volume load are all chromatographic conditions which could be assessed, and this adjustment can improve the analysis time, resolution and sensitivity.

2.2.10.1.1 Particle Size

The size of the chromatographic stationary phase particle impacts SEC efficiency. Compared to bigger particles, these tiny particles have shown to be superior in terms of resolution. Research by Liu at al investigated heavy and light chains using small particle size columns and observed for the effect for a reduced antibody review, resolution improvement and higher efficiencies (Liu et al., 2009). SEC separations benefit from particles with a diameter of 1–2µ m, according to theoretical analyses of the optimal particle size. (Guiochon et al., 1985) Sub-µm SEC column packing materials have made it possible to achieve these resolution enhancements. Columns with different particle sizes were tested for monoclonal antibody analysis by Diedrich. (Diederich et al., 2011) At greater flow rates, shorter run times and better resolution and efficiency were seen for sub-2µm SEC columns.

2.2.10.1.2 Loading a Sample

SEC chromatographic resolution and sensitivity are affected by sample volume and mass load (Fountain et al., 2009). Proteins should be separated according to their molecular weight. Analyte resolution might suffer when the column is overloaded. Sample quantities between

5% and 10% of the entire column volume are ideal for loading. The lower the sample volume, the lower the resolution. Peak distortion is often caused by these higher volumes (Hong et al., 2012).

2.2.10.1.3 Mobile Phase

Secondary interactions may be mitigated by the using modifiers that are organic or variations of additions, like arginine (Yumioka at al., 2010). Protein recovery is typically aided by the usage of these supplements. To avoid interaction with the stationary phase, arginine functions as a binder for the analyte in solution. According to Arakawa's research, the addition of arginine to the mobile phase increased the amount of protein aggregates that could be quantified (Arakawa et al., 2006). Regarding the addition of a mobile phase additive like arginine, several investigations have indicated an improvement in peak form (Ejima et al., 2005). An alteration in the pH of the mobile phase might cause changes in the protein's non-ideal interactions with the stationary phase, which in turn may affect the protein's three-dimensional structure. Based on the link between the mobile phase pH and the protein's isoelectric point, these interactions may be anticipated. Using low ionic strengths as an example, Golovchenko and colleagues showed that at pH values below the protein pI, ion exchange effects occurred, while at pH levels beyond the protein's isoelectric point, ion exclusion occurred (Hong et al., 2012).

2.2.10.1.4 Concentration of Salt

The increase in ionic strength, or mobile phase's salt content, is a popular method for minimising electrostatic interactions in SEC (Hong et al., 2012). Thus, secondary interactions may be reduced, and peak symmetry, retention duration, and quantitation can all be improved because of this. Using this method, Ricker and Sendoval examined a variety of monoclonal antibodies at various ionic strengths and found promising results (Ricker and Sandoval, 1996).

Some antibodies demonstrated a change in retention time and peak shape that was poor using concentrations of sodium chloride that were low, even though the findings differed across the antibodies. It was also shown that electrostatic interactions had an impact on the recovery of synthetic human parathyroid hormone aggregates. Sodium chloride and acetonitrile concentrations had an effect on aggregate recovery in their experiment. It was discovered that adding 100 mM sodium chloride reduced electrostatic interactions and increased aggregate recovery (Hong et al., 2012).

It is usual practice to increase the mobile phase of the solution's counter ion concentration, to reduce electrostatic interactions. Hydrophobic or ion exclusion effects may be enhanced in solutions with large concentrations of these ions (Stulk at al., 2003).

2.2.10.1.5 Flow Rate

The resolution optimisation may be improved by adjusting many factors, one of which is flow rate. Low linear velocities are necessary for optimal column efficiency in many chromatographic separations of macromolecules (Hong et al 2012). SEC may be improved by reducing the flow rate. Ricker and Sandoval examined how the SEC separation of a protein mixture was influenced by flow rate: the improvement of resolution was seen for bovine serum albumin and ovalbumin when flow rate was decreased (Ricker and Sandova, 1996). In denaturing circumstances, Liu et al. investigated how the reduced antibody's SEC separation was impacted by the flow rate. This study demonstrates improvement in resolution are accompanied by broader peaks, longer periods of analysis, and reduced sensitivity (Liu et al., 2009).

2.2.10.1.6 Choice of Column

The peak capacity and resolution may be enhanced by reducing the systems contribution to band broadening and increasing the SEC columns' inner diameter (Hong et al., 2012). If system dispersion is sufficient, it may be necessary to use 7.5 mm I.D. SEC columns to

optimise peak capacity (Diederich et al., 2011). Smaller ID columns (4.6 mm) may now be utilised to obtain equivalent resolution to SEC-HPLC thanks to modern low dispersion instruments such as UHPLC. Increase in the length of the column may provide a way of resolution improvement in isocratic separation like SEC. (Ricker and Sandoval, 1996).

2.2.10.1.7 Instrumentation and Chromatographic Conditions

To preserve the biological function of the macromolecule, SEC separations of proteins have been carried out in their original circumstances. Metal-protein adducts, and unwanted protein interactions can be minimised using biocompatible chromatographic techniques. A physiological pH, a high salt content, and 100 percent aqueous mobile phases are all common requirements for native conditions, although they might be challenging to achieve. High salt concentrations impair system and column performance by increasing the likelihood for particles in the mobile phases. In the absence of bacteriocides, a highly watery mobile phase can lead to bacterial contamination within hours (Link et al.,1985).

2.2.10.1.8 Detector

SEC analysis still relies primarily on UV detection. (Qian et al; 2008) Aromatic amino acids, such as tryptophan, respond better to longer wavelengths, such as those found in the near ultraviolet (Aitken et al., 1996). Far UV or low wavelengths (214 or 220 nm), where the amide peptide bond has a strong absorption, providing higher sensitivity. Either scattering from particles with shorter wavelengths or the presence of chromophores that absorb at 280 nm can lead to quantitative inaccuracies in both wavelength ranges. Each wavelength range, however, has its own advantages: shorter wavelengths are more sensitive, allowing for the study of low-concentration proteins, while higher wavelengths provide a wider dynamic range.

Purity profiling in SEC can benefit from the advantages of both wavelength ranges by adopting dual wavelength detection. Low-abundant species benefit from the shorter wavelength, whereas the more numerous species benefit from a longer wavelength and a wider linear range (i.e., the monomer). The principal species' wavelength ratio is determined empirically. In order to compute the percentage of aggregates and other pollutants that can be detected at a lower wavelength, this factor is utilised. Bond has shown for monoclonal antibodies that this approach allows for lower aggregate levels to be tested against a monomer (Bond et al., 2010)

2.2.10.2 Chromatographic Parameters used for Analysis for Insulin Analysis

2.2.10.2.1 Chromatography Sample

1.5ml from all the formulations were transferred into the HPLC vial and capped for analysis on the chromatography system.

2.2.10.2.2 MAbPac Reversed -Phase TM parameters.

2 µl injection volume at 80◦C (± 0.8 ◦C), was used, a detection UV wavelength was applied from a range of 205 -280 nm for increased detection for a range of insulin. Flowrate was set to 0.3 ml/min for mobile phase at 275 bar maximum column pressure. The data was collected at 20 Hz. The MabPac Rp 4µm 2.1mm x 50mm column from Thermo fisher Scientific was used.

2.2.10.2.3 Mobile Phase Preparation

Two sets of MAbPac Reverse phase TM mobile phase preparation was optimised A1 & B1 (Table 2.4)

Mobile phase A1 was prepare with TFA and water (0.1:100) in 500ml Duran bottle. (100:0.1 % v/v) of TFA was added to a volumetric flask filled with water to the meniscus level and then

inverted several to ensure mobile phase homogeneity. Mobile phase composition termed B1 Mobile phase was prepared with water, Acetonitrile and TFA at a ratio of (90:10:0.1 %v/v). 50 ml volume of water was pipetted into a 500ml volumetric flask, adding the acetonitrile and 0.5 ml TFA prepared in a volumetric flask to the meniscus level. Note that small portions of the mobile phase (approx. 5-10ml) was used to rinse the Duran bottle before the prepared mobile phase was transferred. Furthermore, the bottle containing the solution was inverted several times to ensure mobile phase homogeneity.

Table 2.4. MAbPac reversed - phase TM column optimized gradient

Time (minutes)	Composition A1(%)	Composition B1(%)
0	100	0
1	100	0
11	0	100
12	0	100
14	100	0
15	100	0

2.2.10.3 Chromatographic Parameters used for Analysis for IgG Formulations

2.2.10.3.1 Chromatographic sample

1.5ml from all the formulations were transferred into the HPLC vial using 0.2µm filter and capped for analysis on the chromatography system.

2.2.10.3.2 Column Parameters

The SEC analysis was carried out with 1.5 µl volume injection at 300 µml/min flow rate, using ACQUITY UPLC Protein BEH SEC column 200 A 1.7Um, 4.6 x 300 mm, 1/pkg (01883426017072) Waters (UK). A wavelength of 280nm was utilized at 20 min run time and

a temperature of 50 ℃ in both column compartments (left and right). 214 nm, 220 nm, and 280 nm DAD wavelengths were selected and applied.

2.2.10.3.3 Mobile Phase Preparation

0.2M potassium phosphate buffer at pH 6.8 containing 0.25M potassium chloride was used for SEC analysis. The buffer was filtered with 0.2µm and transferred into a clean Duran bottle after being rinsed with buffer solution.

2.3 Data Analysis

- Origin 7.0 Origin DSC analysis software
- Microsoft software (excel spread sheet)

2.4 Statistical Analysis

One way ANOVA was used to determine the significant difference of a group of data where significant levels are expressed in p-values. The P-values less than 0.05($P < 0.05$) is considered significant while ($P > 0.05$) is considered insignificant.

CHAPTER THREE

3.0 INFLUENCES OF BETA CYCLODEXTRIN AND PLURONIC F-127 ON SPRAY DRIED AND ELECTROSPRAYED LYSOZYME AS A MODEL PROTEIN

3.1 Introduction

Protein medications are typically manufactured as aqueous dosage forms, which are administered intravenously (Ohtake et al., 2011). Liquid dosage forms on the other hand, are typically unstable and have a short shelf life (Mensink et al., 2017) as they tend to precipitate, clump, or separate out if the formulation conditions are incorrect (Moussa et al., 2016). This necessitates the storage and transportation of medications in refrigerated settings. When compared to liquid protein formulations, the process of delivering and storing protein in a dried state or powder form has several advantages. These advantages include a lower rate of degradation, a longer shelf life, and the elimination of the need for cold storage (Butreddy et al., 2021). It is possible to synthesize protein medicines in a dry powder form to circumvent these constraints (Dimitrov, 2012). The therapeutic protein will remain stable and active under the desired controlled conditions for the duration of the stated storage period (three years or longer) when stored in powder form at room temperature (Pinto et al., 2021). An additional drying step (spraydry and electrospray) is frequently included in the manufacturing of protein therapeutics to counteract protein instability caused by the solution state. The native structure of a protein can be preserved during long-term storage by dehydrating the liquid protein formulation into a powder form first (Kaspere and Friess, 2011; Butreddy et al., 2021).

In the spray drying process, protein structure and bioactivity are improved by adjusting the formulation and processing parameters (Ji et al., 2017). Traditional tiny drug molecules, which are simpler in structure and more stable, are not adequate to stabilize the complicated structure of biopharmaceuticals to ensure the safety and efficacy of administration. (Wang, 2015; Faber & Whitehead, 2019). A three-dimensional structure also known as the native state is essential for proteins to be pharmacologically active, the native state formation is fragile based on weak

physical interactions such as ionic bond, hydrogen bond and Van der Waal attractions, making it unstable to a certain degree (Hulse et al., 2008). The failure of this structure decreases drug potency, pharmaceutical activity and therapeutic impact. In order to compensate for the lack of stability in the treatment, protein medicines are commonly manufactured as solids (Hulse et al., 2008). The preparation of solid protein formulations by drying proteins is another strategy for combating the limited stability of proteins and achieving an acceptable shelf life as pharmaceutical products (Abdul-Fattah and Truong, 2010, Wang, 2000). However, drying processes and storing proteins in a dried state can also be damaging to proteins, and stabilising excipients are typically required in order to protect against the drying stresses (Chang and Pikal, 2009). Dehydration is not only a drying method that can be used to extend the shelf life of proteins, but it can also be used to create protein particles for use in a variety of delivery routes (Ohtake et al., 2011). Several studies have shown that dried biopharmaceutical powders are suitable for use in inhalation preparations for the nasal, pulmonary and sustained drug delivery systems (Langford et al., 2018). Dehydration is a simple and cost-effective method of preparation (Mensink et al., 2017; Wang et al., 2012) of dried therapeutic protein powders. These dried therapeutic protein powders have demonstrated excellent storage stability at room temperature.

The manufacture of dried biopharmaceuticals is limited by parameters such as manufacturing time, temperature, and a variety of process-related stresses due to proteins being sensitive to various environmental stresses (Maa et al., 1999; Emani et al., 2018). Spray drying process produces suitable small fine particles making it acceptable for engineering due to its adaptability, cost-effectiveness, simplicity and scalability. (Fourie et al., 2008; Sou et al., 2011). The use of this method could produce protein powders with high stability (Andya et al., 1999)

Spray drying can be optimized by changing formulation composition and processing conditions to ensure the structural integrity and bioactivity of proteins. Also, addition of

excipients in the formulation may improve the stability of lysozyme during spray drying (Ji et al., 2017). New methodologies for identifying the impact of excipients on protein stability are needed to improve biopharmaceutical formulations. (Collins, 1997; Falconer, 2019; Kalayan et al., 2021) Lysozyme is a stable, tiny protein with molecular weight of 14.4kDa that has been intensively investigated and observed to remain mostly folded in both experiments and simulations.

Lysozyme was employed as a model protein, because it is a protein that has been well-studied and better understood, as a result, it is more vulnerable to destabilization from a variety of forces, including aggregate formation, deamidation, and oxidation (Merlini & Bellotti, 2005; Singh & Singh, 2003). Furthermore, lysozyme is a catalyst for hydrolysis of Micrococcus. luteus, which occurs roughly 108 times faster than the uncatalyzed process (Voet & Voet, 1995;Varman & Singh, 2012). As a result, it is best-suited for finding out how beta cyclodextrin and pluronic F-127 influences its biological activity. To also explore the effect of these excipients on lysozyme's conformation stability and biological activity, in this study.

This chapter will investigate the effect of excipients (pluronic F-127 and beta cyclodextrin) on lysozyme formulation and help to understand how lysozyme is stabilized with these excipients. The biological activity of spray-dried lysozyme formulation with and without excipients will be assessed, the thermal stability of lysozyme will be investigated. This chapter is organized following a brief introduction, the methods of the experiments used are described in chapter 2 in the method section. The results and discussion section describe the observation during the experiment and conclusions are presented at the end.

3.2 Objectives

The challenges faced in the pharmaceutical industry on protein stability is enormous. Drying methods and addition of excipients have been identified as ways to improve this protein stability. The objectives of this chapter are:

- To study the effects of drying on Spray-dried (SD) and Electrosprayed (ESD) lysozyme formulations with and without excipients using various characterization techniques like differential scanning calorimetry (DSC) to determine the thermal stability with lysozyme solid samples, dynamic light scattering for particle size and scanning electron microscope (SEM) for morphology of the dried particles.
- To observe the effect of the technique (SD and ESD) on lysozyme biological activity using UV-Vis spectroscope for enzymatic activity assay.
- To assess the effect of temperature on native lysozyme formulations in dry and solution forms with and without excipients.

3.3 Effects of Drying Techniques (Spray-drying and Electrospray) on lysozyme formulations with and without excipients

The effect of the techniques (Spray dried and Electrosprayed) was observed by studying various characteristics such as percentage of protein yield, biological activity, particle morphology and particle size examination.

3.3.1 The Percentage Yield Determination of Spray-dried Lysozyme (SD) and Electrosprayed (ESD) Formulation

The process for the determination of the percentage yield of lysozyme after drying using electrospray and spray drying method is outlined in this section.

A detailed method of the process for the determination of the percentage yield was outlined in Chapter 2 section 2.2.3.1, the sample collected after drying was weighed and expressed in percentage.

Table 3.1 shows the percentage yield of the dried sample using spray dry and electrospray techniques with and without 1%w/v beta cyclodextrin, 1%w/v and 5%w/v, pluronic F-127, all in low concentration.

Table 3.1: Percentage yield of dried formulation with and without excipients using drying techniques n=1

Formulation	**Yield (%)**
1%w/v SD LYSOZYME	29
1%w/v SD LYSOZME WITH 1%w/v BETA CYCLODEXTRIN	53
1 %w/v SD LYSOZYME WITH 5%w/v PLURONIC	30
1%w/v SD LYSOZYME WITH 1%w/v BETA CYCLODEXTRIN AND 5% w/v PLURONIC	50
1%w/v ESD LYSOZYME	30
1%w/v ESD LYSOZYME WITH 1%w/v BETA CYCLODEXTRIN	25
1%w/v ESD LYSOZYME WITH 1%w/v PLURONIC	41
1%w/v ESD LYSOZYME WITH 1%w/v BETA CYCLODEXTRIN AND 1%w/v PLURONIC	4.9

The powder yield ranged from 4.9-53%. A 29% spray-dried formulation was achieved showing a relatively low yield. 53% of spray-dried power with the addition of beta cyclodextrin was achieved. The powder recovered with the addition of 1% (w/v) beta cyclodextrin was clearly higher with a significant percentage compared to spray-dried lysozyme without excipient. The addition of beta cyclodextrin yielded a promising result because it reduced the deposition of the dried lysozyme on the wall of the spray drying chamber and has a better influence because a powder yield is dependent on inlet temperature and the atomizing gas flow rate. On the other hand, the addition of pluronic F-127 produced a reduced yield on the formulation as a 30% yield was obtained. Reduced powder yield can be attained when the temperature of amorphous powder reaches a sticky temperature and when there is insufficient drying before it impacts the walls of the drying chamber, these impact results in particles adhering to the walls of the dryer.

Pluronic is observed to be viscous. A sticky point may be dependent on temperature glass transition (Tg) and the moisture distribution in the dried particle, Lipianen et al (2018) suggest that sticky behavior could be obtained when process temperature exceeds the material Tg by 10-20 degree approximately. A product yield of 30 to 40% is normally expected with the help of the bench-top spraying system (Hulse et al., 2009; Bowen et al.,2013). Moreover, in the pharmaceuticals, production on a large scale of spray-dried pharmaceutical drugs is possible with the use of a large scalable spray drier, which results in the best possible yield. This is an advantage for the industry. The spray drying technique has a problem with poor particle collection of the small size particles, which has a significant effect on the final product's percent yield. Because of this, particles that have a low density have a chance of being dragged up into the vacuum of the spray drier (Prinn et al, 2002; Haj-Ahmad et al., 2013). The possibility to further improve powder recovery lies with technically improving the design of

the dryer (Bowen et al.,2013) and also, optimizing process parameters such as inlet temperature, drying time, pressure, gas-flow rate and solvent used.

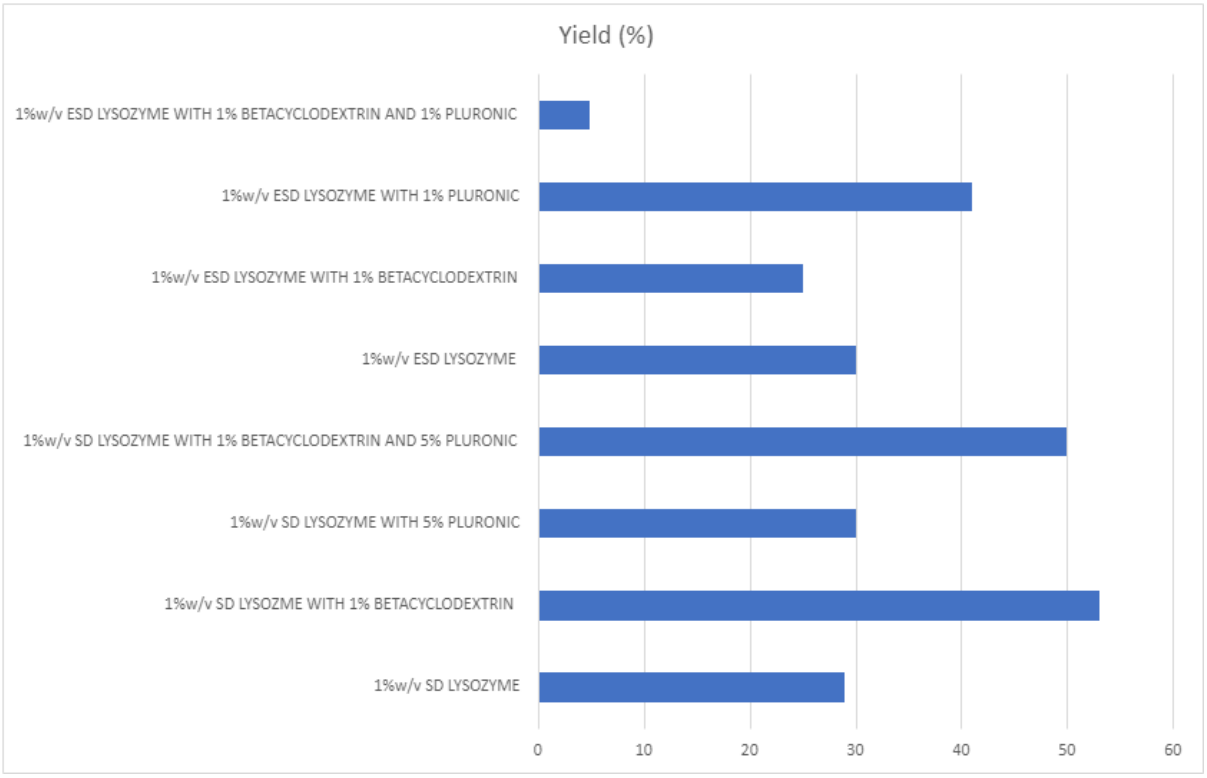


Figure 3.1 A chart showing the percentage yield of dried formulation in the presence and absence of excipients using spray drying techniques.

The bar chart in Figure 3.1 gives a further clear representation of the percentage yield. Both drying techniques showed various significant effect on the formulations. Dried (SD, ESD) lysozyme formulations without excipients produced a low percentage yield of proteins (29%, 30%) compared to the formulations with excipient. The percentage yield for SD sample with beta cyclodextrin produced higher yield (53%) compared to electrospray formulation with beta cyclodextrin producing only (25%). ESD sample with pluronic F-127 at 1%w/v produced more yield compared to 5%w/v pluronic of SD formulation.

A major loss was observed for electro sprayed lysozyme with the addition of a combination of excipient. 50% percentage yield was observed for spray dry lysozyme with a combination of beta cyclodextrin and pluronic F-127 compared to electro spray formulation with 4.9% yield.

The powder yield is dependent on the inlet temperature and the atomising gas flow rate for spray dryers, with the atomising gas flow rate having a stronger influence on the formulation, these parameters affect the drying and temperature of the product. Reduced powder yield can be observed when drying is not sufficient before impact with the drying chamber wall or when the temperature of amorphous powders reaches the so-called sticky point temperature, also resulting in a particle adhesion to the walls of the cyclone dryer (Maury et al., 2005; Lipiainen et al., 2018). Addition of beta cyclodextrin to ESD Lysozyme formulation produced no significant change with (25%) reduction compared to ESD without excipient (30%). Lu et al., (2007) reports that protein formulation stability depends on percentage type of the excipient used in the formulation. A major concern when adopting spray drying is the poor product yield, which is 30–40 percent in a benchtop system (Maa et al., 1998). Therefore, an increased percentage yield can be achieved by optimising the drying conditions during spraying and through the introduction of a polymeric excipients with a high glass transition temperature.

Furthermore, the produced particle's physical properties can be affected by a variety of process parameters, such as the amount of water being fed into a nozzle, voltage supply and distance between nozzle tip and collecting platform (Abraham et al., 2019). Spray-drying formulations can be produced in big quantities in the pharmaceutical industry by using a large scalable spray-drier that can produce the best potential output while for electrospray, utilising a multi-tip emitter, for example can be done to increase the possibility upscale electro spraying (Abraham et al., 2019). It is interesting to note the time required by each method to produce the quantity shown in Table 3.1 was clearly observed. The spray-drying process took 15–20 minutes for the entire sample, whereas the electrospray sample took more than six hours to complete using the same sample volume (Abraham et al., 2019).

3.3.2 Effects of Beta Cyclodextrin and Pluronic F-127 on the Particle Size of Spray-dried Lysozyme Powder

In the determination of the particle size distribution of lysozyme formulations, Polydispersity index (PDI) is a parameter used to define the size range and populations within sample formulations. The values numerically varying from 0.0 – 1.0 where 0.0 signifies perfect sample uniformity with respect to the particles size and 1.0 indicates that the sample is highly polydisperse with multiple particle size populations (Danaei et al., 2018). Table 3.2 shows the mean size and PDI of spray dry and electrospray formulations in this study. The particle size of the formulation ranges from 2.206µm to 4.329µm and PDI ranging from 0.223 to 1.000.

Table 3.2 showing the mean size and PDI in standard deviation of spray-dried and electrospray formulations. n=3

PROTEIN FORMULATION	**PDI**	**Size (µm)**
1%w/v UNPROCESSED LYSOZYME	0.223± 0.161	4.329±1.471
1%w/v SPRAY-DRIED LYSOZYME	0.253± 0.06	3.478 ± 0.06
1%w/v ELECTROSPRAY LYSOZYME	0.936± 0.033	1.642± 0.196
1%w/v SPRAY-DRIED LYSOZME WITH 1%w/v BETA CYCLODEXTRIN	0.251±0.393	2.943±0.209
1%w/v ELECTROSPRAY LYSOZME WITH 1%w/v BETA CYCLODEXTRIN	1.000±0.000	3.066±0.932
1%w/v SPRAY-DRIED LYSOZME WITH 1%w/v BETA CYCLODEXTRIN AND 5%w/v PLURONIC	1.000±0.000	3.124±1.117
1%w/v ELECTROSPRAY LYSOZME WITH 1%w/v BETA CYCLODEXTRIN AND 1%w/v PLURONIC	1.000±0.000	2.554±0.521
1%w/v SPRAY-DRIED LYSOZME WITH 5%w/v PLURONIC	1.000±0.000	2.701±0.927

1%w/v ELECTROSPRAY LYSOZME WITH 1%w/v PLURONIC	0.989±0.020	2.206±0.673

***PDI: Polydispersity Index**

PDI measures the breadth of the molecular weight distribution of the samples by describing how much material is present in each of the different molecular weight segment. The PDI values of formulation obtained in this research shows most of the formulations to be higher indication of heterogenous size distribution in the sample. DLS technique measures the size distribution of particles which is the property of interest (Bera, 2015; Danaei et al., 2018). PDI from 0.2 and below are stated as acceptable for nanoparticles polymer-based samples, values lower than 0.05 are seen mainly as monodispersed while values higher than 0.7 shows very broad particle size distribution (Danaei et al., 2018).

The spray dry powder size, using dynamic light scattering (DLS) were found to be significantly affected by the excipients. Adding 1%w/v beta cyclodextrin and 5%w/v pluronic F-127 to the lysozyme solution before spraying appeared to have decreased the particle size as seen in Table 3.2 with the unprocessed lysozyme at 4.329µm and spray-dried lysozyme below 3.478µm. The ideal aerodynamic particle size distribution of particles intended for pulmonary delivery has been determined to be between 1 to 5µm (Hickey, 2016). As a result, all particles produced in this study fall within the same range and are therefore suitable for pulmonary delivery. About 80% of particles smaller than 3 microns have a possibility of entering the lower airways, and 50–60% of them end up in the alveoli (Labiris and Dolovich, 2003) The pulmonary route is increasingly being used by pharmaceutical researchers as a reliable method of systemic medication administration. It is fast becoming more and more well-liked as a quick and efficient method of administering medications both locally and systemically to the lungs (Siew, 2014).

Enhancements in device technology have improved the patient-friendliness and convenience of inhaled medicine delivery. More importantly, from a therapeutic perspective, the lungs offer a high absorption region with significant vascularization, resulting in quick drug delivery. Additionally, compared to the oral route, lesser doses can be delivered because it avoids first-pass metabolism (Siew, 2014). First pass metabolism is described as drug metabolism whereby the concentration of a drug when administered orally is greatly reduced before it reaches systemic circulation example.

3.3.3 Microscopic Examination of Spray-dried and Electrosprayed Lysozyme using Scanning Electron Microscopy

The morphology of the spray-dried lysozyme was studied using scanning electron microscope (SEM). The spray-dried lysozyme with 1%w/v of beta cyclodextrin and 5%w/v of pluronic F-127 were observed with a description of the method detailed in chapter 2 in section (2.2.9.2). The SEM micrographs are presented in Figure 3.2 showing the morphology of the various formulation particles.

The surfaces of all spray-dried particles shown by the scanning electron microscopy appeared smooth spherical and uniform except for beta cyclodextrin with dimples with surface diameter between 5-5.7micrometer.

Spray drying showed a practical way to make fine particles, regardless of the excipients used. In relation to the size of geometric particles, all the spray-dried formulations had the particles under 5 micrometres in size in volume diameter using DLS in Table 3.2. It was discovered that the particle size was dependent on excipient and concentration and scanning electron micrographs did not exhibit a gross difference in particle morphology with different excipients however, exhibited a different morphology with spraying technique (Figure 3.2 & 3.3).

All the surfaces of spray dry lysozyme showed a fused spherical appearance except beta cyclodextrin with a dimpled doughnut shape similar to research conducted by Maa et al (1996) using a different type of sugar (trehalose).

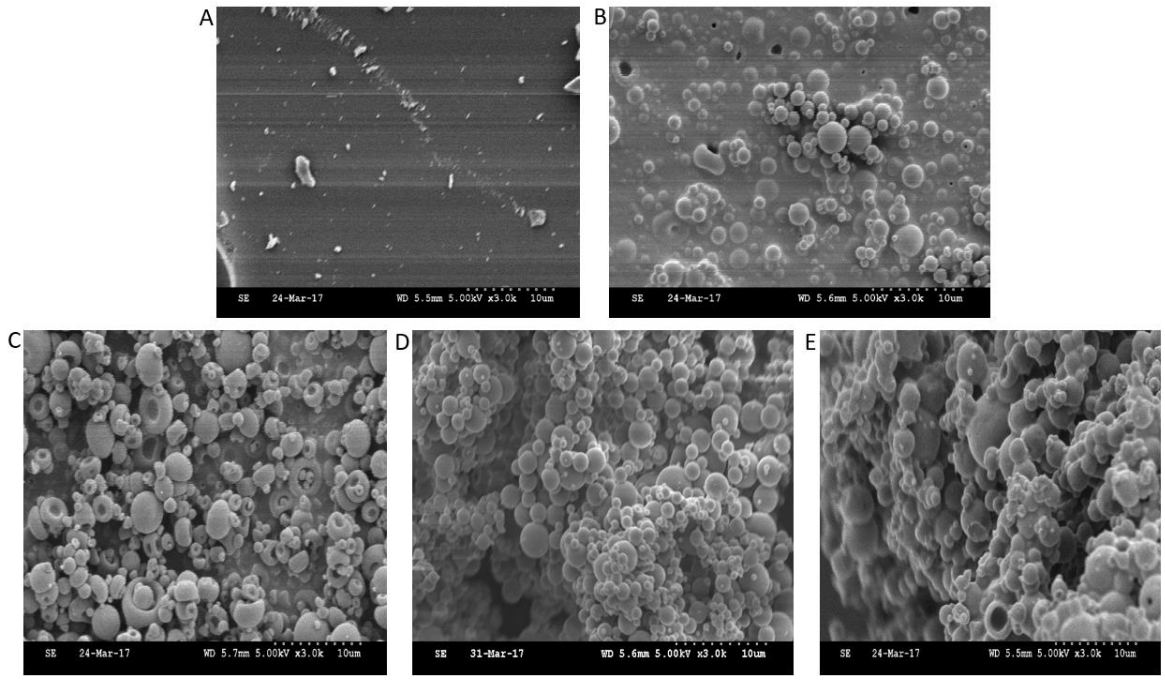


Figure 3.2 SEM images of unprocessed lysozyme and spray-dried lysozyme. (A) Unprocessed Lysozyme without Excipients; (B) 1%w/v Spray-dried Lysozyme (C) 1%w/v Spray-dried Lysozyme with 1%w/v Beta cyclodextrin; (D) 1%w/v spray-dried Lysozyme with 5%w/v Pluronic (E) 1% w/v spray-dried Lysozyme with 1%w/v Beta cyclodextrin and 5%w/v Pluronic.

Rate of drying has also been observed to impact spray dried particle, the drying rate is important because faster drying is more likely to result in dimpled dried particles than slower drying. The quick evaporation of the liquid from the center of the spherical particle results in holes if the surface is solid and crusty otherwise, holes may occur if water escapes by diffusion from the particle (Wang and Wang, 2002). Adding excipients to the spray-dried protein particles is confirmed to improve the shape of the particles in this investigation except beta cyclodextrin (Figure 3.2C) as dimples have been observed.

A microscopic examination of electrospray lysozyme was further conducted using SEM. The SEM images for electrospray powder containing beta cyclodextrin and pluronic F-127 are

shown in Figure 3.3. The powders were amorphous and dry. Amorphous materials produced by different drying techniques have different physical properties, such as particle size, structure, and surface area (Faldt and Bergenstahl, 1996). Morphology is usually affected by the process of protein preparation, processing technique and the type of excipients used. All the protein formulations exhibited different shapes, forms and sizes using the same condition. Unprocessed lysozyme appeared flaky, Spray-dried lysozyme produced smooth and spherical particles compared to unprocessed and electrospray lysozyme. Electrospray showed an impact on the lysozyme producing a needle shape particle. The addition of pluronic F-127 to spray dried lysozyme SEM image produced a round shape with a smooth surface (Figure 3.2D) though agglomeration was observed while electrospray lysozyme showed elongated particles (Figure 3.3C). This could be due to the sticky nature of pluronic. Dried formulations containing high concentrations of sticky surfactants like pluronic are not appropriate for aerosol applications. Redispersibility would be poor due to the high degree of particle agglomeration.

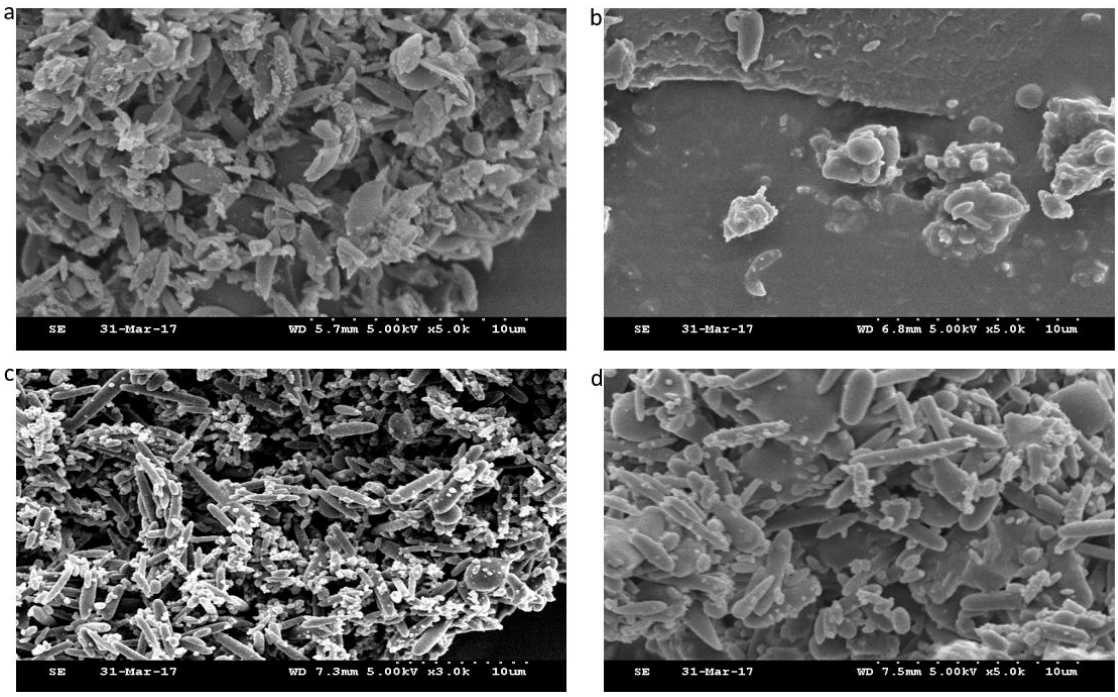


Figure 3.3 SEM images of electrospray lysozyme. (a) 1%w/v electrospray lysozyme without Excipients (b) 1%w/v electrospray lysozyme with 1%w/v beta cyclodextrin; (c) 1%w/v electrospray lysozyme with 5%w/v pluronic (d) 1%w/v electrospray lysozyme with 1%w/v beta cyclodextrin and 5%w/v pluronic.

Various factors are responsible for the difference in surface morphology. At standard conditions, outlet temperature is constant at approximately 52-54˚C (Maa et al., 1997). The nature of surface crust like the formation of dry crust from different types of protein may differ in their flexibility, porosity and mechanical strength, also the liquid droplets are subjected to surface turbulence and interior motion after atomization. Maa et al (1997) went further to research on surface forces to explore how they affect surface morphology and resolved that particle mobility and surface tension suggest that crust properties may be important as concentration of protein to excipient seems to affect the morphology profile, Maa et al (1997) observed particles obtained from spraying protein and mannitol at higher solid concentrations were denser in the dry droplet, he further stated the denser the structure is, the more likely it is to be more resistant during spraying. This investigation explains the reason for a shallow dimple observed from a higher solid concentration. A reduction in the concentration of beta cyclodextrin may be considered in subsequent research as a more spherical particle size is desired for drug administration. Spray-dried protein particle morphology was found to be substantially influenced by operational and formulation variables. In the spray drying process, the main critical parameter was the output temperature, which indicates drying speed. The sphericity of the resultant particle was improved by using a lower outlet temperature. Depending on the type of sugar and surfactant present, the protein formulation had a significant impact on particle morphology.

The electrospraying setup employed in this study include a conducting needle using same voltage. At the needle's tip, a stable Taylor cone is usually formed, resulting in relatively homogeneous particles with distinctive morphology. The integrated protein disperses evenly throughout the solution (both with and without the excipient), enhancing the amorphous nature and bioavailability (Mehta at al., 2018).

The particle size of the electrosprayed particles has a strong influence on the flow rate. It was observed that higher flow rates result in lower particle sizes. The flow rate utilised in this

investigation was regarded low, which could explain the high particle size of electrosprayed lysozyme, particularly when beta cyclodextrin was used (Abraham et al., 2019). Gomez et al (1998) proposed lowering protein concentration in the solvent or the flow rate to achieve a smaller particle size using electrospray technique. An obvious challenge connected with excipient is that excipients are often used to regulate the protein-protein interactions (PPIs) in formulations but complicated by the fact that the design of excipients is not well understood, and the different components of these systems are made up of transient, weak interactions among proteins, excipients, ions, and water in solution where this issue remains an extension of the problems that have long been associated with understanding aqueous electrolytes, particularly those of simple salt solutions, whose behavior at increasing concentrations is poorly understood (Kamerzell et al., 2011).

3.3.4 Effect of Drying Technique on Lysozyme Powder Formulations using Thermogravimetric Analysis.

The moisture content of lysozyme powder formulation was analysed using a Malvin Mettler Toledo (UK) instrument. Refer to chapter 2 section 2.2.8.1 in the method section of chapter two for a detailed method applied for this analysis. Figure 3.4 shows the various percentage weight loss of the lysozyme powder formulations. The unprocessed lysozyme exhibited a loss of moisture content of 3.7%, spray dried lysozyme showed 0.5% and electrospray lysozyme was 3.4%. Spray dried lysozyme formulation showed a lower weight loss compared to other formulations which showed a higher weight loss.

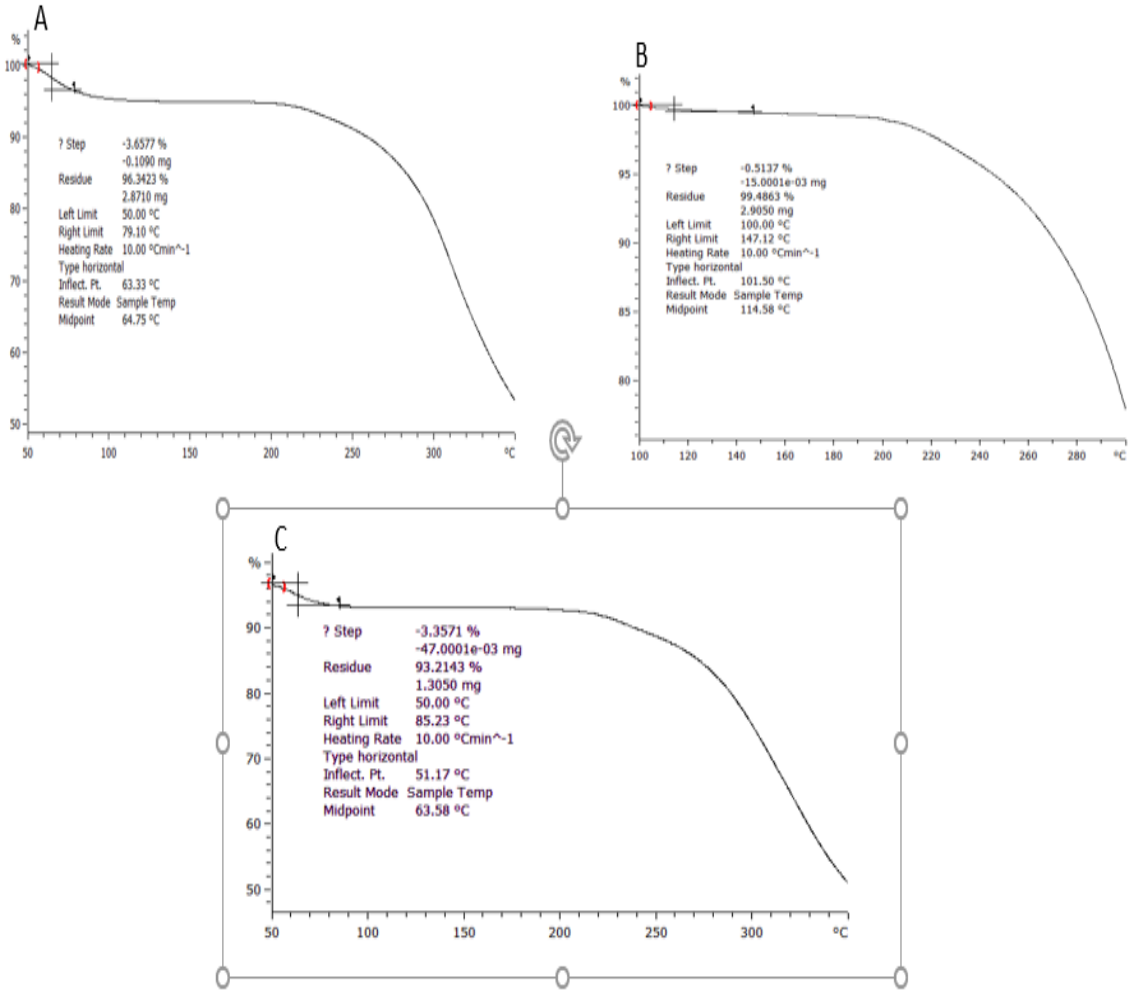


Figure 3.4 TGA curve for (A) 1%w/v unprocessed lysozyme, (B) 1%w/v SD lysozyme, (C) 1%w/v ESD lysozyme

The spray-dried sample showed a slight change in the weight of the sample compared to the unprocessed and ESD lysozyme. The weight of all other samples was constant from about 100 °C to about 200°C indicating the lysozyme samples is thermally stable to about 200°C**,** from this temperature it indicates there is a loss of weight as decomposition starts at the said temperature. Moisture content has a significant impact on biopharmaceutical products' solid-state stability, with increased rates of decomposition beyond a monolayer. Temperature and composition of the drying processes are important variables, as water can act as a plasticizer and redistribute water available to the protein. An accurate moisture determination is essential for resolving stability issues (Town, 1995).

3.4 The Effect of Drying Technique (Spray dry and Electrospray) on Lysozyme Activity in the Presence and Absence of Excipients

3.4.1 Effects of Beta cyclodextrin and Pluronic F-127 and Drying Technique on the Biological Activity of Lysozyme Formulations.

Micrococcus lysodeikticus buffered suspension is cloudy with a high intensity absorbance at 450nm. The bacterial cell wall is ruptured by lysozyme, resulting in cloudy suspension clearing which indicates a reduction in absorbance. As a result, measuring the rate of decrease in absorbance at 450 nm of *M. lys suspension* indicates lysozyme biological activity. The biological activity of nine (9) formulation are outlined in the Table 3.3 and further details in Appendices v showing three (3) different time intervals (60,120,180) seconds. The samples after drying were reconstituted and the biological activity was expressed as a percentage relative to the protein control. Both drying techniques with and without excipients provide protection for the activity of protein except spray dry lysozyme formulation with pluronic showing a reduced activity. At 60 seconds, SD and ESD lysozyme showed 90- 100% activity and remained active, maintaining the activity of the protein.

Table 3.3: Showing the biological activity of Unprocessed, spray dried and electrosprayed formulations.

FORMULATION	**% BIOLOGICAL ACTIVITY**		
	60sec(1min)	**120 sec (2mins)**	**180 sec (3min)**
1%w/v UNPROCESSED LYSOZYME	100	100	100
1%w/v SPRAY-DRIED LYSOZYME	112.3	90.1	50.9
1%w/v ELECTROSPRAYED LYSOZYME	91	113	108
1%w/v SPRAY-DRIED LYSOZYME WITH 1%w/v BETA CYCLODEXTRIN	92.63	87.06	75.45

1%w/v SPRAY-DRIED LYSOZYME WITH 1%w/v BETA CYCLODEXTRIN AND 5% PLURONIC	95.79	122.8	112.6
1%w/v SPRAY-DRIED LYSOZYME WITH 5%w/v PLURONIC	69.01	38.83	48.5
1%w/v ELECTROSPRAYED LYSOZYME WITH 1%w/v PLURONIC	91	92	111
1%w/v ELECTROSPRAYED LYSOZYME WITH 1%w/v BETA CYCLODEXTRIN AND 1% PLURONIC	103	98	92
1%w/v ELECTROSPRAYED LYSOZYME WITH 1%w/v BETA CYCLODEXTRIN	96	100	89

Enzymatic activity assay was carried out to observe the effect of excipients on the biological activity of lysozyme after spraying drying. Tables 3.3 & 3.4 show the biological activity results of spray-dried lysozyme and represent the biological activity of the reconstituted lysozyme solution with and without the added excipients (Pluronic F-127 & Beta cyclodextrin). The Spray drying process maintained high activity (90-100%) of the lysozyme at 120 seconds. Preserved activity was also observed in the spray-dried lysozyme formulation containing beta cyclodextrin, showing greater compatibility with lysozyme and maintaining the biological activity of the protein. This suggests that the reaction conditions did not affect the biological activity of spray-dried lysozyme with the addition of beta cyclodextrin compared to that of pluronic. The addition of pluronic F-127 showed a decreased ability to hydrolyze the bacterial cell walls. Although non aqueous solvents tend to destabilize proteins, certain type of solvent with low lipophilicity can have the reverse effect, stabilizing proteins at low concentrations (Wang, 1999), pluronic have a polar, water-soluble group linked to a nonpolar water-insoluble hydrocarbon chain. A folded protein in an aqueous environment tends to have hydrophobic sections shielded from hydrophilic parts exposed to the water. An aqueous solvent's polarity reduces when a nonaqueous solvent is added to it, and protein hydrophobic cores may disperse into the solvent, disrupting the protein hydration shell and causing instability and protein

unfolding (Varman and Singh, 2012). This better explains the effect of pluronic on biological activity as the PEO block has a hydrophilic nature and can dissolve in water, whereas the PPO block has a hydrophobic nature and cannot dissolve in water. These block copolymers, when placed in an aqueous environment, will self-assemble into micelles. These micelles will have a hydrophobic PPO central core and a hydrophilic PEO outside shell that will interact with water (Ottenbrite and Javan, 2005). Therefore, the properties of pluronic may have destabilized lysozyme resulting in decreased biological activity. ESD lysozyme with pluronic F-127 and beta cyclodextrin provided protection for protein activity and maintained about 90% activity. Beta cyclodextrin provided protection for spray-dried lysozyme compared to SD lysozyme with pluronic showing a reduced activity. This could be due to a higher inlet temperature or aspirator rate used during spraying causing the pluronic powder to melt and stick to the walls of the cyclone.

A combination of beta cyclodextrin and pluronic F-127 provided protection for protein activity during spray drying and electrospray. The protein activity for all formulations with excipients were between 90-100% confirming the stability effect of the excipients except SD lysozyme with pluronic F-127. It could be certain that a combination of beta cyclodextrin and pluronic F-127 could increase the activity and stability of protein using the right technique and formulation as Hulse et al., (2008) outlined excipient interacting with lysozyme to stabilize it against any loss of activity. The 3-D structure also known as tertiary structure of protein is the essential component for biological activity and should be intact. Any disruption involving the linkage that holds the tertiary structure in place could result in a loss of enzymatic activity and deformation (Ghaderi and Carlfos, 1997). Following further characterization, using the kinetic mode of the uv-vis spectroscopy. The result obtained from basic and kinetic method showed similarities. Although there are a few discrepancies.

It is safe to state that the addition of beta cyclodextrin maintained the activity and stability of protein during the biological activity investigation. Excipients have been shown to interact

with lysozyme in a way that prevents any potential loss of activity, according to research carried out by Hulse et al. (2008). Moreover, the amount of water that is present on the active site cleft is another factor that determines how well it works. According to the findings of Nagendra et al. (1998)'s research, the active site of the lysozyme molecule is a highly hydrated region, and the presence of around 0.2 grammes of water per gramme of protein is required for lysozyme to possess biological activity. The author proposes further that the active-site cleft of the protein should be hydrated by at least 9.4 percent moisture content. If this is not done, the active-site cleft will contract, which will result in the protein becoming inactive. Beta cyclodextrin might have played a significant role in hydrating lysozyme's active site region. It also could have supplied a good substrate fit, which would have prevented the inactivation of lysozyme due to the drying procedure and would have kept the biological activity intact (Abraham et al., 2019).

Table 3.4: Showing the data obtained from biological activity of the formulations with kinetic mode using UV-VIS spectroscope.

FORMULATIONS	**% BIOLOGICAL ACTIVITY**		
	60sec(1 min)	**120 sec (2mins)**	**180 sec (3mins)**
1%w/v UNPROCESSED LYSOZYME	100	100	100
1%w/v SPRAY-DRIED LYSOZYME	85	86	89
1%w/v ELECTROSPRAYED LYSOZYME	98	103	103
1%w/v SPRAY-DRIED LYSOZME WITH 1%w/v BETA CYCLODEXTRIN	99	99	96
1%w/v SPRAY-DRIED LYSOZME WITH 1%w/v BETA CYCLODEXTRIN AND 5% PLURONIC	75	78	82
1%w/v SPRAY-DRIED LYSOZME WITH 5%w/v PLURONIC	58	63	67
1%w/v ELECTROSPRAYED WITH 1%w/v PLURONIC	90	95	96

1%w/v ELECTROSPRAYED WITH 1%w/v BETA CYCLODEXTRIN AND 1% PLURONIC	Aggregated		
1%w/v ELECTROSPRAYED WITH 1%w/v BETA CYCLODEXTRIN	93	96	96

The decreased activity of pluronic F-127 on spray-dried lysozyme could be as a result of the high inlet temperature or the aspirator rate used. Both inlet and aspirator rate could contribute to the outlet temperature making the inlet temperature have a stronger impact on the protein. The outlet temperature can be thought of as the highest temperature to which the finished product is subjected to (Cal and Sollohub, 2010). The other protein activity was not affected by the atomising gas flow. Maintaining biological activity is very crucial in any protein formulation because it gives an indication about the foldability, integrity, and stability of protein. It is reported that higher pressure atomisation results in stronger mechanical stress and can be harmful to protein but no evidence of this was observed in beta cyclodextrin. This stabilizing effect of pluronic F-127 and beta cyclodextrin evaluated in this work is evident when compared to other excipients researched in previous study by Lipiainen et al., 2018; Elkordy et al., 2004; Mensink et al., 2017). Excipients are confirmed to provide full biological activity during spraying via process setting adjustments. Spray-dried lysozyme with inulin reported by Ronkart et al., (2009) preserved 100% of the biological activity as an amorphous sugar with a high glass transition temperature of 155 °C. The results obtained from this study explains the importance of adding excipients to spraying process as it causes perturbation of the tertiary structure of the native lysozyme thereby decreasing its biological activity. Kheddo et al suggest pH of a solution may impacted the biological activity, structure and stability of proteins (Kheddo et al., 2014) These results agree with Elkordy et al. (2002) and Hulse et al. (2008).

3.5 Effect of Temperature on Lysozyme Formulation in Dried and Solution Form with and without Excipients

3.5.1 Thermal Stability Studies of Lysozyme Formulations in Solution Form using High Sensitivity Differential Scanning Calorimetry (HSDSC)

High Sensitivity Differential Scanning Calorimetry also referred to as Micro-cal VPDSC monitors thermal stability of reconstituted lysozyme preparations to detect thermal denaturation. The degree to which a protein's structure is protected from thermal denaturation is known as the structural stability of the protein. Denaturation temperature obtained at the thermal transition temperature is calculated at the thermogram peak midpoint and the area under the peak known as the calorimetric enthalpy. During heat absorption, a peak appears when there is a transition from a folded state to an unfolded state, which is then characterized by the thermodynamic parameters Tm. The endotherm obtained details the mode of curve fitting procedure, notation and definition of various thermodynamic parameter illustrated in the principles of operation of VP HDSC in chapter 1 and the method section. Water-water and buffer-buffer run were carried out on the instrument to check its efficiency. The sample and reference cells containing buffer alone were up scanned at a constant heating rate. The respective baseline was subtracted and used for analysis and the conditions used for the experiment is also explained in the method section. The data obtained respectively from the DSC were analysed using non two state cursor fitting model.

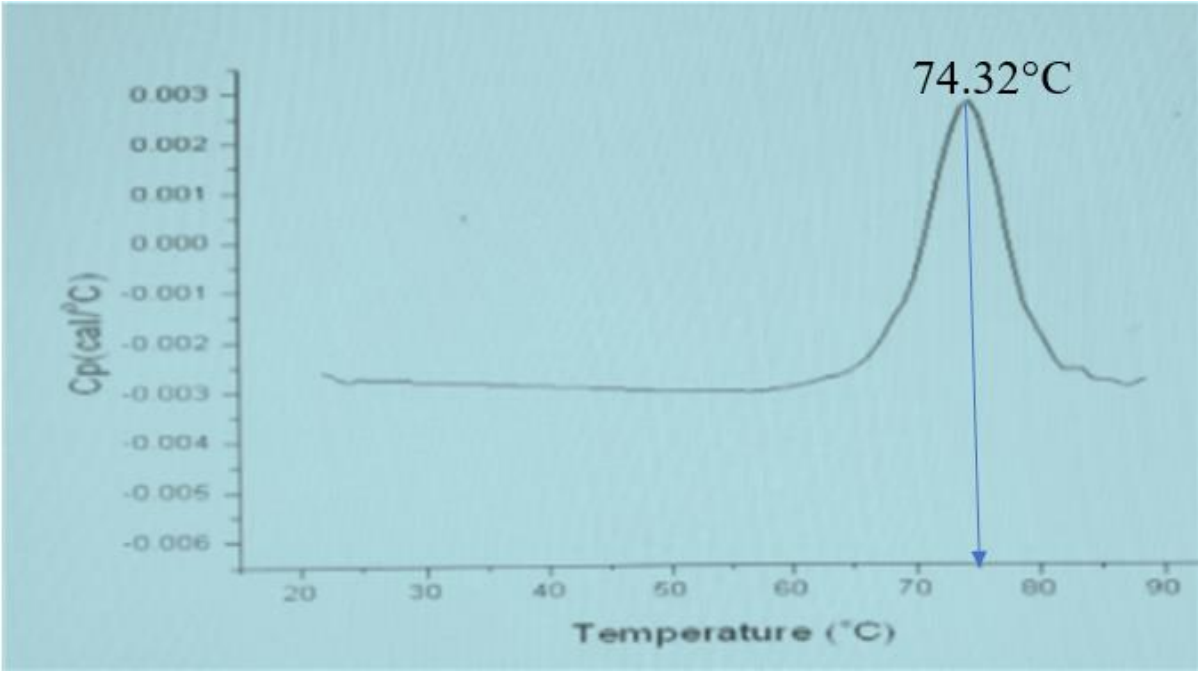


Figure 3.5 Represents a DSC thermogram of unprocessed lysozyme solution. 5mg/ml in phosphate buffered saline at pH 6.24

The denaturation temperature of unprocessed lysozyme with and without excipients in the absence of a drying technique showed a negative effect on the denaturation temperature. The addition of excipient in the absence of drying in this study has not shown any improved stability of lysozyme as shown in Table 3.5. However, showed minimal effect. The thermograms obtained during the analysis (picture not included) showed a transitions for all formulations beginning with the unprocessed 1%w/v lysozyme showed endoderm transition with a single peak at T_m=74.32°C ± 0.16, 1% spray-dried lysozyme showed single peak at T_m=72.46°C ± 0.38, 1%w/v electrospray lysozyme showed a single peak at T_m=74.44°C ± 0.22, 1%w/v spray-dried and electrospray with beta cyclodextrin showed T_m=74.36°C ± 0.16 and T_m=74.55°C ± 0.5 respectively. Figure 3.5 shows the raw thermogram of unprocessed lysozyme melting under certain conditions. A Tm of 74.32°C was observed with 0.006M phosphate buffer at a pH of 6.24.

Table 3.5 Showing the midpoint transition temperature in standard deviation of freshly prepared formulation without drying technique. n=2

Formulation	**Denaturation Temperature T_m (°C)**
1%w/v UNPROCESSED LYSOZYME	74.32±0.16
1%w/v LYSOZME WITH 1%w/v BETA CYCLODEXTRIN	73.32±0.19
1 %w/v LYSOZYME WITH 5%w/v PLURONIC	73.74±3.4
1%w/v LYSOZYME WITH 1%w/v BETA CYCLODEXTRIN AND 5%w/v PLURONIC	73.36± 4.3

Table 3.6 displays the results of the thermal analysis conducted on unprocessed, spray dry (SD), and electrospray (ESD) lysozyme with and without excipients using high sensitivity DSC with the Tm of the scan from the DSC thermograms see appendix viii for raw DSC data. Spray-drying in the absence of excipient showed a decreased thermal stability compared to the unprocessed lysozyme and other formulations. Electrospray lysozyme in the absence of excipient maintained thermal stability. Spray dry and electrospray lysozyme with excipients maintained thermal stability however, aggregation was observed for electrospray lysozyme after 8 months of storage. HSDSC is used extensively to study the effect of formulation components on the conformational stability of proteins. Midpoint transition temperature (Tm) also known as denaturation temperature and are thermodynamic parameters used in HSDSC to analyze protein stability during the component formulation as well as excipient interaction. Information are provided via these measurements on protein folding and stability (Elkordy et al., 2004; Cooper and Johnson, 1994). HSDSC solution Tm was used to investigate protein

denaturation and the effect of the spray drying technique on the conformation stability of protein in lysozyme solution.

Table 3.6 Showing the midpoint transition temperature of lysozyme formulation in the presence and absence of beta cyclodextrin and pluronic f-127 and the effect after 8 months of storage n=2

Formulation	**Denaturation**	**AFTER 8 MONTHS STORAGE**
	Temperature Tm(°C) (Solution form)	
1%w/v UNPROCESSED LYSOZYME	74.32±0.16	
1%w/v SD LYSOZYME	72.46±0.38	73.48±0.1463
1%w/v SD LYSOZME WITH 1%w/v BETA CYCLODEXTRIN	74.36±0.26	73.61±0.4484
1%w/v SD LYSOZYME WITH 5%w/v PLURONIC	74.81±030	74.12±0.2042
1%w/v SD LYSOZYME WITH 1%w/v BETA CYCLODEXTRIN AND 5%w/v PLURONIC	74.23±1.90	74.53±0.354
1%w/v ESD LYSOZYME	74.44±0.22	Aggregated
1% w/vESD LYSOZYME WITH	74.51±0.52	Aggregated

1%w/v BETA CYCLODEXTRIN		
1%w/v ESD LYSOZYME WITH 1%w/v PLURONIC	74.29±0.45	Aggregated
1%w/v ESD LYSOZYME WITH 1%w/v BETA CYCLODEXTRIN AND 1%w/v PLURONIC	64.31±0.86	Aggregated

A lower denaturation temperature (72°C**)** was observed in the spray-dried formulation (Table 3.6). Addition of beta cyclodextrin and pluronic F-127 during spraying maintained the stability of lysozyme. A source of stress such as an increased atomization rate could affect spray drying leading to degradation of the protein (Maa et al., 1997). Other factors such as feed flow rate, inlet temperature, aspirator capacity and nozzle flow rate may have affected the spray drying result. (Stahl et al., 2002). Addition of excipients to the spray-dried formulation is observed to maintain the functionality of the protein. The conformational stability was more stable with the addition of excipient as well. Spray drying seems to affect the thermal stability of protein thereby affecting the functionality. After storage, the spray-dried formulation appears to increase stability however, this appears unreliable as several research conducted records spray-dried formulations without excipient are less stable. It has been recorded and confirmed by several researchers like Elkordy et al., 2002 that preparation of protein using spray drying technique usually requires addition of excipients to reduce the protein from some deterioration. Lipiainne et al., (2018) outlines spray-dried protein formulations commonly require stabilising excipients to prevent protein degradation during processing and storage

confirming the results obtained in this research. Addition of excipient without drying in this study did not show significant stability of lysozyme as p value> 0.05 was observed.

3.5.2 Thermal Stability Studies of Lysozyme Formulations in Solid Forms using Differential Scanning Calorimetry (DSC) in the Presence and Absence of Beta cyclodextrin and Pluronic F-127

In analyzing the stability of lysozyme in the presence of its formulation components and its interaction with excipients, thermodynamic parameters such as Tm were utilized. It was used in this examination to investigate the effect of beta cyclodextrin and pluronic F-127 on conformational stability of lysozyme.

The result of the thermal profiles obtained from unprocessed, spray dry and electrospray are outlined in Table 3.7 showing the apparent denaturation temperature values of the protein sample with and without excipients. The figures represent the flow of heat as a function of temperature. See appendix iv for analyzed thermograms.

Table 3.7 Thermal analysis showing apparent denaturation temperature Tm (˚C) of spray-dried lysozyme in the presence and absence of beta cyclodextrin and pluronic F-127.n=1

PROTEIN FORMULATION	**DENATURATION TEMPERATURE (°C)**
1%w/v UNPROCESSED LYSOZYME	199.45
1%w/v SPRAY-DRIED LYSOZYME	201.30
1%w/v ELECTROSPRAYED LYSOZYME	222.18

1%w/v SPRAY-DRIED LYSOZME WITH 1%w/v BETA CYCLODEXTRIN	-------
1%w/v SPRAY-DRIED LYSOZME WITH 1%w/v BETA CYCLODEXTRIN AND 5% PLURONIC	-------
1%w/v SPRAY-DRIED LYSOZME WITH 5%w/v PLURONIC	----------
1%w/v ELECTROSPRAYED WITH 1%w/v BETA CYCLODEXTRIN	225.94
1%w/v ELECTROSPRAYED WITH 1%w/v BETA CYCLODEXTRIN AND 1% PLURONIC	225.49
1%w/v ELECTROSPRAYED WITH 1%w/v PLURONIC	223.26

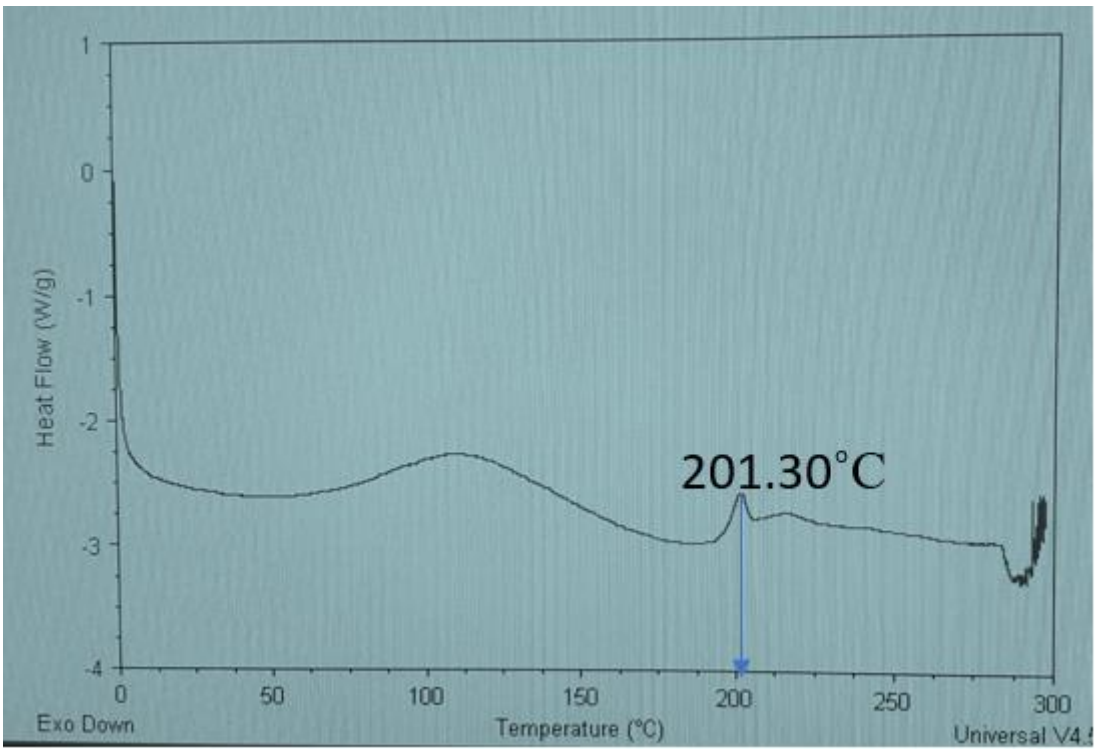


Figure 3.6: Showing thermal denaturation of spray-dried formulation at a midpoint temperature of 201.30 ˚C

Spray-dried formulations from the DSC analysis showed a broad endotherm at 100˚C and a clear peak (Figure 3.6) while spray-dried lysozyme with excipients (beta cyclodextrin; pluronic F-127) showed the absence of a peak which might be related to heat induced interaction of the polymer and drug or solubilization of the excipient in the melted state. It could also be that the heat and mechanical stress may have impaired the integrity and viability of protein during the heat and atomization process respectively. Martias et t al, (1991) in their work refer to samples degrading due to heat because heat is described as the physical stress majorly affected by the protein as well as evaporation of water. Further, the drying process involving dehydration could also be a factor responsible for the structure of the protein as the removal of water molecules from the surface of some protein can also destabilize the protein (Liao et al., 2002). This result confirms research published by (Andya et al., 1999; Namaldi et al., 2006; Tzannis and Prestrelski, 1999. The temperature used during the drying process could have affected the integrity of the protein containing the excipients because temperature is a

factor that affects drying process in spray drying technique. The temperature that is used in the drying process should be high enough to allow for efficient drying, but it should not be so high that the protein's viability and integrity are jeopardized. In addition, it is impossible to rule out the possibility of mechanical stress occurring during the atomization process, tests need to be carried out to ensure that there will be no reduction in activity. The biological activity illustrated in Table 3.3 for these same formulations showed that the lysozyme is still active after undergoing the drying process. It could be that the protein is still active while some parts of the structure may be affected as the thermograms with excipients have not shown any transition. It is also possible to suggest that disulphide bond was not affected by disulphide exchange in the protein. Though it is documented that disulphide exchange causes a reduction in biological activity while intermolecular disulphide exchange can result in aggregation of individual polypeptide molecule.

Two or more broad endothermic peaks were observed at 100˚C on all the DSC thermograms. The first endothermic broad peak in Figure 3.6 indicates an increase in water content present in the preparation (Hulse et al., 2008; Abraham et al., 2019). The second peak (endothermic) at about 200˚C shows the apparent denaturation temperature transition of the protein. Elkordy et al., (2002) also reveals that second exothermic temperature indicates denaturation, and the broadly observed exotherm could be due to an internal heterogenicity in the characteristics of the protein molecules (Fan and cooper, 1994).

The drying techniques (spray-dry and electrospray) tend to improve denaturation temperature of lysozyme as compared to unprocessed lysozyme, although the transition for spray-dried lysozyme appeared not significant with a 2˚C difference compared to 22˚C for electrospray lysozyme. Addition of beta cyclodextrin and pluronic tend to improve and increase the denaturation temperature for electrospray lysozyme when compared to spray-dried lysozyme with excipients.

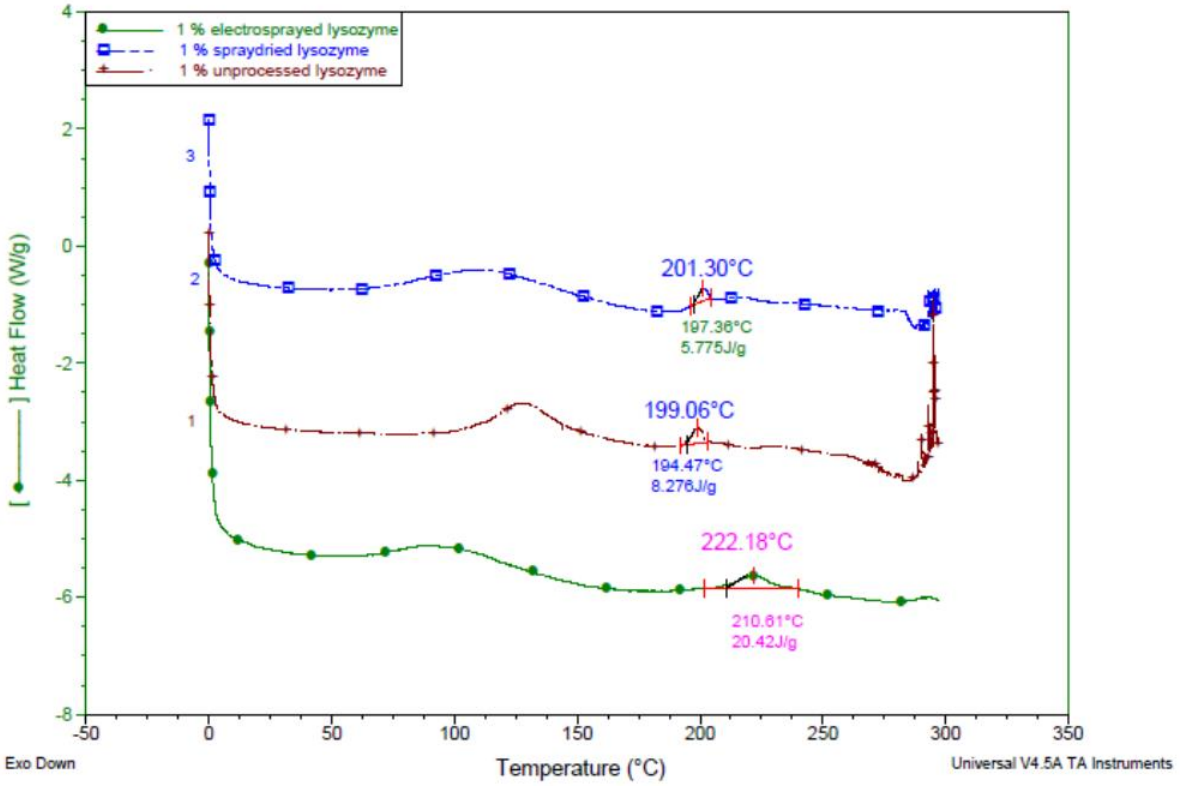


Figure 3.7. DSC thermogram of unprocessed, spray-dried and electrospray lysozyme in the absence of Beta cyclodextrin and Pluronic F-127

Electrospray technique improved the apparent denaturation temperature of the lysozyme solution making electrospray lysozyme stable with denaturation temperature at 222.18 ˚C compared to spray drying at 199.06 ˚C (Figure 3.7). Furthermore, electrospray lysozyme formulation with excipients suggests an increase in thermal stability as observed from the denaturation temperature (Figure 3.8), as the addition of pluronic F-127 exhibited a denaturation temperature of 222.43ºC, this indicates that addition of excipient to the electrospray lysozyme maintained the stability of the protein.

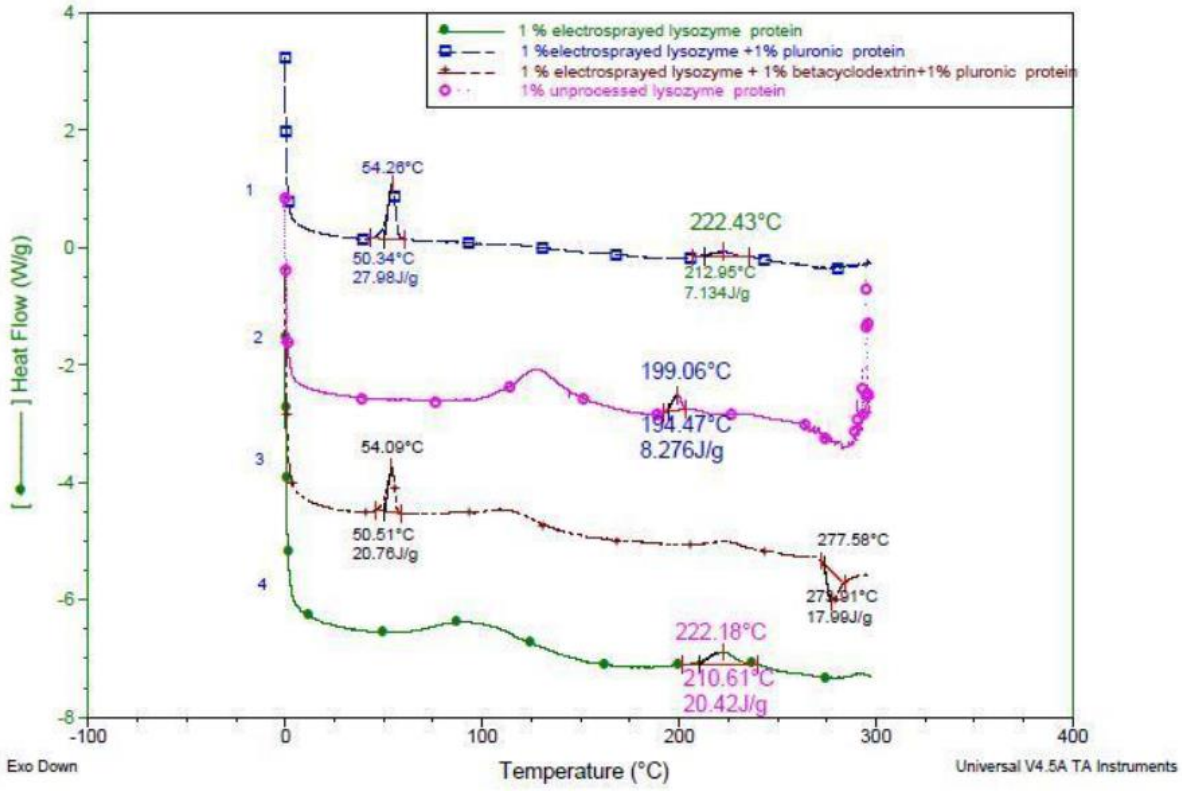


Figure 3.8 DSC Thermogram of Unprocessed and Electrospray Lysozyme with Betacyclodextrin and Pluronic F-127

The increase in temperature for electrospray confirms it is more stable compared to spray-dried and unprocessed lysozyme at one month. However, the ESD samples all got aggregated over 8 months storage period (Table 3.6), this may suggest that moisture in the protein could have a negative effect on electrospray samples. Gill and colleagues (2010) stated in their findings that higher denaturation temperature causes the molecules to be more stable. Hence, high apparent denaturation temperature for electrospray lysozyme with excipient (beta cyclodextrin and pluronic) appears more stable as they exhibited an increase in denaturation temperature. Interestingly, biological activity confirms the result as lysozyme showing 90 to 100% activity.

3.6 Conclusion

Drying technique together with the addition of excipients have been observed to improve stability and biological activity of protein.

This chapter has observed the effects of drying techniques (Spray drying and Electrospray) on lysozyme formulations.

The ability of DSC to measure changes in the conformational structure of spray-dried lysozyme was demonstrated in this research, the spray-dry temperature, the type and number of excipients employed throughout the procedure are both important in preserving spray-dried formulations.

Formulations with beta cyclodextrin and pluronic F-127 have shown some promising qualities with the drying techniques applied as some formulations containing both excipients have exhibited a form of stability looking at the enzymatic activity.

Addition of beta cyclodextrin to spray dry lysozyme improved and maintained the biological activity. Also, addition of beta cyclodextrin and pluronic F-127 maintained the denaturation temperature of spray dry lysozyme.

Spray dried technique in the absence of beta cyclodextrin and pluronic F-127 impacted the denaturation temperature in a negative way. It could be stated that a combination of beta cyclodextrin and pluronic could increase the activity and stability of protein using the right technique and formulation as the electrosprayed technique has shown.

Spray-dried lysozyme with excipients showed some specific actions in the biological activity, however, it showed lack of stability using the DSCs apparent temperature. Studies show that proteins can have some activity to a certain level while part of the structure might have been affected by degradation or dehydration. As such, degradation of lysozyme was not ruled out in this study as heat could have been a major physical stress that affected the formulations as outlet temperature used for spraying was 73°C The drying technique improved the thermal denaturation of lysozyme also, both technique and excipient could enable drying operations with satisfactory powder yields being achieved in their unique forms. Beta cyclodextrin appeared to be a better excipient to maintain stability and activity of lysozyme formulation in

this study. It also presented an advantage of more efficient drying with spray dry techniques compared to electrospray.

Spray dry formulation analysis using HSDSC with and without excipients remained physically stable after months of storage compared to electro sprayed lysozyme as most formulations appeared to be aggregated after reconstitution.

Pluronic F-127 and beta cyclodextrin demonstrated several promising qualities with electrospray and spray-dried lysozyme formulations, physical stability of the amorphous products including efficacy in protein stabilisation and efficiency in spray dry and electrospray processes However, consideration should be given to the advantages and disadvantages of each drying approach when making a sensible decision on the drying procedure used to improve the stability of therapeutic proteins for various drug administration applications.

CHAPTER FOUR

4.0 EFFECT OF BETA CYCLODEXTRIN AND PLURONIC F-127 ON STABILITY OF INSULIN IN SPRAY DRIED AND SOLUTION FORMS

4.1 Introduction

Protein solutions such as insulin is mostly faced with aggregation challenges and the inability to formulate protein and peptide drugs into solid state. The instability of protein drugs in solid dosage forms is one of the reasons the products are not administered through oral route rather, it is administered through intravenous and subcutaneous route (Wang, 1999). Insulin is currently administered by subcutaneous injections. It is also administered via pulmonary and nasal routes (Nuffer et al., 2015). The main concern with systemic injection is that only a limited percentage of insulin reaches the liver with success for physiological activity (Wang et al., 2015). Sometimes oral delivery is not possible as some types of oral dosage forms cannot protect the drug from the proteolytic breakdown in the gastrointestinal tract and the harsh acidic environment (Shargel, 1999; Xing, 2003). Parenteral delivery route avoids the biological barriers that prevent the entry of proteins through biological membranes. Insulin is an example of drugs administered through this route. Solid formulations are produced through spray drying. Spray drying has a high throughput, and the characteristics of spray dried particles can be modified to obtain desired flowability (Maa and Prestrelski, 2000; Mutukuri et al., 2021).

Inhalable insulin product such as Exubera (withdrawn from market) and a fibrinogen powder combination called Raplixa are both made by spray drying (white et al., 2005). A span of 1.5 to 2 years is termed to be the desirable shelf-life period for protein drugs regardless of storage temperature. Currently, insulin prescriptions are stored in refrigerators to maintain the efficacy and safety of the product (Carpenter et al., 1997). Various stabilising techniques are being researched to improve stability. An approach that can be successful in the delivery of oral

proteins is through their protection from acidic gastric environment associated with low pH in the stomach.

4.2 Aim

As it is stated in various research carried out that addition of additives and controlled scan rate helps in the improvement of insulin stability, the aim of this chapter is to determine ways to improve the stability of insulin formulations through drying method (spray dry) with the help of mini-Buchi spray dryer, and to investigate the thermal denaturation of insulin in different states.

4.3 Objectives

- To prepare dried insulin formulations with and without excipients using beta cyclodextrin and pluronic F-127.
- To characterize different states of insulin through the determination of the effect of temperature on insulin stability using different scan rates at 10 and 60^{o}C/hr. with various techniques like Differential scanning calorimetry (DSC), Dynamic light scattering (DLS), Scanning electron microscope (SEM).
- To investigate the thermal denaturation of insulin.
- To check the effect of 1% (w/v) excipient pluronic F-127 and 1 %w/v beta cyclodextrin, on native insulin.
- To explore the effect of HCl and Phosphate buffered saline on insulin stability and comparison of marketed insulin with native insulin.

4.4 Result and Discussion

4.4.1 Determination of Percentage Yield for Spray dried Insulin Formulations

The spray dry (SD) insulin sample yield was obtained by using the percentage yield formular and method described in Chapter 2 (section 2.2.3). Figure 4.1 illustrates the percentage yield

of 1% (w/v) insulin formulation in the presence and absence excipient (Pluronic F-127 and Beta cyclodextrin) obtained after drying the insulin formulations in form of a bar chart.

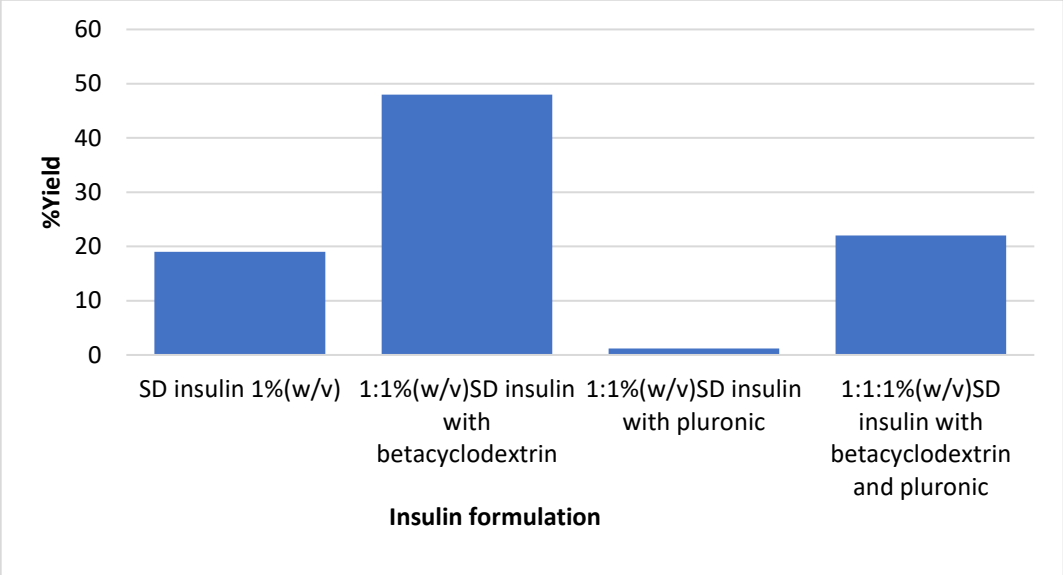


Figure 4.1: Percentage yield of insulin sample after spraying with a spray dry technique. n=1

The percentage yield shows SD insulin with pluronic, experienced the most sample loss because most of the powder were disintegrated on the wall of the cyclone. However, addition of beta cyclodextrin to SD insulin decreased the deposition of the sprayed powder on the walls of the cyclone and the drying chamber. As a result, Beta cyclodextrin showed a higher percentage (48%) compared to SD powder without excipient. On the other hand, Pluronic F-127 had difficulty giving a good spray. This significantly affected the percentage yield of the formulation. In spray drying method, temperature has an impact on the stability of protein. Degradation of protein could be affected by an increase in air atomisation rate during spraying due to protein adsorption at the air/liquid interface of the protein solution (Maa et al., 1998; Lo et al., 2004). Another limitation encountered is the problem of efficient particle collection after spraying resulting in low percentage yield. Smaller particles with lower masses cause them to be drawn into the vacuum compared to samples with higher particles. The result

observed for SD insulin with Pluronic F-127 justifies the low yield (Prinn et al., 2002). The cyclone has a potential to capture larger particles hence the high percentage yield of insulin with beta cyclodextrin. Furthermore, stress generated during the spraying process include high temperature which may negatively affect the drying process if not properly controlled, thereby, affecting the stability and the structure of the proteins (Manning et al., 2010; Mutukuri et al., 2021). This condition may have affected insulin with pluronic F-127 due to the viscous nature of the surfactant. Spray drying of protein may lead to denaturation of protein because elevated temperatures are needed for efficient drying. Nevertheless, it can be minimised by circulation of water around the solution containing the protein if not, high residual moisture content might be observed in the protein formulation (Haj-ahmad et al., 2013). Pluronic F-127 may not be a good excipient for use during spray drying because of its viscous nature, Other drying methods may be advised because excipients react differently with different proteins, hence the choice of additive. Processing technique and controlling processing conditions may influence the stability of protein.

In terms of applications, while pluronics are known to inhibit surface–tissue adhesion for many cell types they have been successfully used for scaffolding (Klouda and Mikos, 2008).

4.4.2 Microscopic Examination and Particle size of Spray-dried Insulin

Different morphologies were observed for the spray dried powders, the SD insulin formulations and the interaction between the excipients could be explained in the particle morphology. Figure 4.2 shows the micrograph of insulin powder in the presence and absence of beta cyclodextrin and pluronic F-127. The micrographs exhibited different irregular morphology, some of the particles appeared to have rough surfaces and some particles showed slight cavity and cracks. Irregularities affecting morphology of particles could be as result of the rate of water evaporation during the spray drying process for most proteins. However, this may not be true as pure insulin in this study has shown similar morphology with the other

formulations with excipients. Faster evaporation is attained at a high temperature creating a defined and smoother surface.

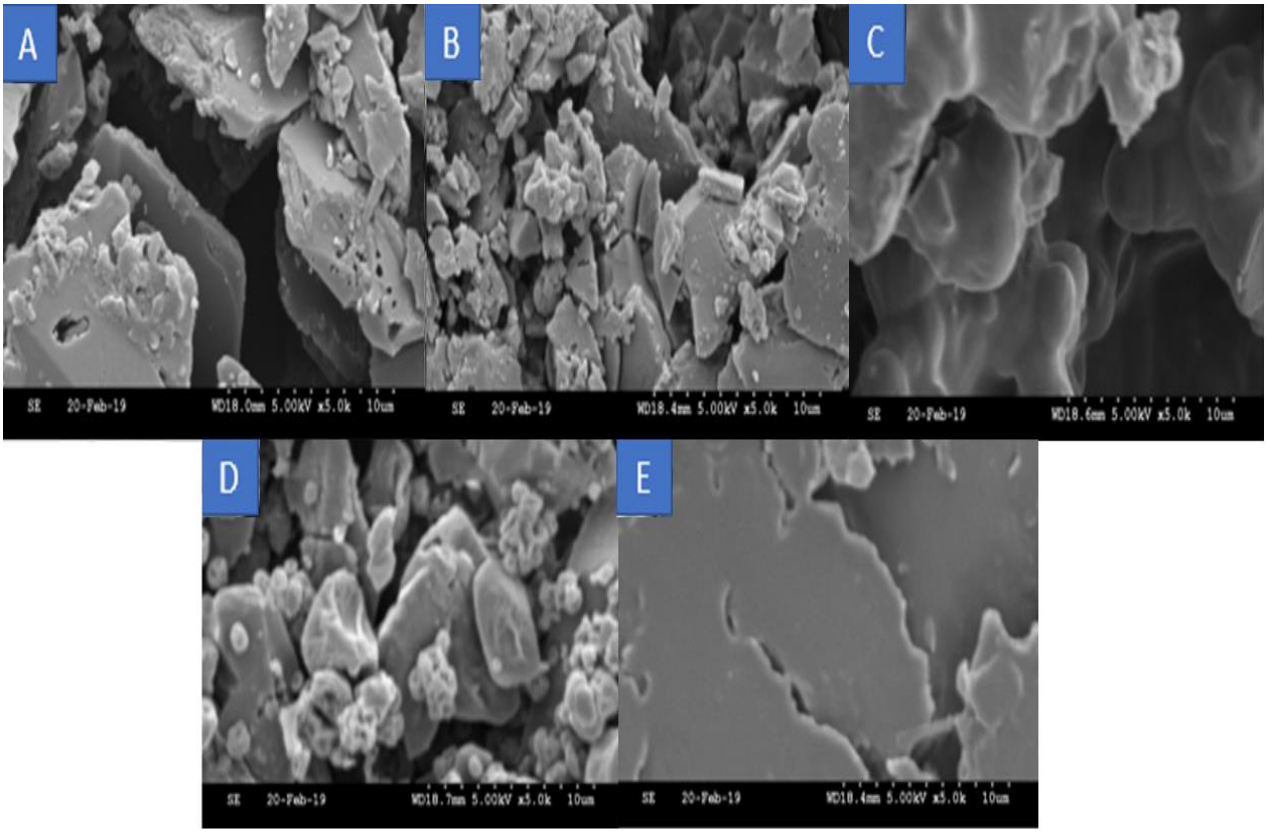


Figure 4.2 showing the SEM IMAGES (a) Pure insulin, (b) SD insulin (c) SD insulin beta cyclodextrin pluronic, (d) SD insulin beta cyclodextrin, (e) SD insulin with pluronic.

The SEM pictures in Figure 4.2 appears to be more of sheets rather than smooth particles with is usually expected, Figure 4.2 (A) shows larger sheets, (B) irregular smaller sheets with very tiny particles, (C). rough surfaces, (D) blocks with tiny, fused particles, (E) large plain sheet.

The irregularities observed could be as a result of the in-let temperature applied in the drying process. The inlet temperature used for drying ranged between 110-130 degrees. It is important to note that lower temperature was considered to preserve the integrity of the protein.

Alamilla-Beltran et al investigated the effect of inlet temperature. It was revealed that lower air inlet temperatures result in irregularity of the shape of microparticles with creased surfaces

while a higher inlet temperature results in a more rigid microparticles and porous surfaces. It was further revealed that spray dry processes at 160, and 180 degrees at a feed rate of 10ml/min produced semi- spherical and more defined particles without evidence of particle agglomeration or cracks. However, in the same experiment conducted, at a feed rate of 8ml/min, the microparticles showed irregular surfaces and particle agglomeration (Alamilla-Beltran et al., 2005). A similar result was outlined by Tonon et al for acai juice encapsulation with maltodextrin. smooth spherical and irregular semi-spherical particle was obtained (Tonon et al., 2009).

Distribution of particle size of insulin particle in the presence and absence of excipient was studied. The particle size ranged from 2193nm to 4053nm on dynamic light scattering. The particles also appeared too heterogenous. Small particles tend to form uniform arrays in solutions with minimal gaps among particles, whilst large particles on the other hand can fill a surrounding gap producing mostly homogeneous solutions that are stable under mechanical flow (Santiago-Adame et al., 2015).

Table 4.1: Analysis of particle size of the spray dry insulin formulation in mean standard deviation, n=3

Insulin formulation	**Particle size mean ± SD**	
	Size (d.nm)	**PDI**
Unprocessed insulin 1%(w/v)	4053 ± 1039	0.907±0.208
SD insulin 1%(w/v)	2117 ±527.1	1.000±0000
1:1%(w/v) SD insulin with beta cyclodextrin	3823± 855.0	0.850±0.197
1:1%(w/v) SD insulin with pluronic F-127	3617±1232	0.951±0.099
1:1:1%(w/v) SD insulin with beta cyclodextrin and pluronic	2193±782.1	0.931±0.097

Since the scattering intensity of a protein in solution is proportional to the square of its molecular weight, DLS is thought to be sensitive to detecting protein aggregation produced by processing procedure. (Hulse and Forbes, 2011; Haj-Ahmed et al., 2013). The results obtained from the DLS are outlined in (Table 4.1). The spray particles in the presence of excipients showed that they all had a mean particle size below 5µm, the particle size analysis showed spray drying of insulin led to a particle size of 2µm. The presence of excipients (Beta cyclodextrin and Pluronic F-127) in the spraying process showed a particle size of more than 3µm. Spray dry insulin with and without excipient showed acceptable particle size range contrary to report by Patel et al. (2001) where it was reported that spray-dried insulin in the absence of excipients did not reach the respiratory size range after spraying. However, report by Patel et al appears to be plausible looking at the SEM images in Figure 4.2.

The range in size is similar to what would be expected to target the lungs which is the proposed site of lungs absorption for insulin, although it appears that these particles are in the relevant size range, requiring further formulation (and possibly size reduction) to achieve deep lung deposition. The spray-dried insulin reached a respirable size range both in size and morphology compared to the unprocessed insulin. Filtering the formulations using 0.2µm filter further helped in the deduction of the particle size of the formulations (Table 4.2) since the presence of large particles may be produced by particle agglomeration process due to aggregation and particle-particle interaction (Santiago-Adame et al., 2015).

Table 4.2: ***Particle size mean standard deviation*** *of filtered insulin sample after spray drying n=3*

Insulin formulation	**Particle size mean ± SD**	
	Size (d.nm)	**PDI**
Unprocessed insulin 1%(w/v)	592.0 ± 78.42	0.542±0.075
SD insulin 1%(w/v)	777.3 ± 103.7	0.720±0.126
1:1%(w/v) SD insulin with beta cyclodextrin	1144± 398.2	0.719±0.246
1:1%(w/v) SD insulin with pluronic	740.7±222.2	0.639±0.167
1:1:1%(w/v) SD insulin with beta cyclodextrin and pluronic	1189±1782.8	0.931±0.097

PDI: Polydispersity Index

4.4.3 Solubility of Insulin

Insulin is observed to be less soluble at neutral pH. It can be solubilised at 1-10mg/ml in dilute acetic or hydrochloric acid at a pH of 2-3. Insulin powder can be frozen and stored at -20°C protected from moisture.

Prescribed insulin solution can generally be stored for up to 12 months at 2 to 8 °C according to pharmacopeia. The solubility of insulin is dependent on several factors which include the temperature and concentration of salts and metal ion and the nature of solvents and pH. Insulin has an isoelectric point of 5.3 and it is almost insoluble to dissolve in neutral conditions. Phosphate buffer saline and HCl are known to be approved buffer and solvent to aid the stability of insulin and were therefore considered in this insulin investigation. In this research,

different concentrations of hydrochloric acid at different molarity were tested and finally arrived at a concentration of 0.1 M HCl to dissolve the insulin powder at a pH of 1.2. Although, it is known that insulin is poorly soluble in phosphate buffer however, 7mM phosphate buffer at pH 7.6 with stirring technique dissolved the human insulin. Minamoto et al suggests alkaline stock solutions are not recommended since high pH increases the rate of deamidation and aggregation (Minamoto et al., 1991).

4.4.4 Storage Stability

All the insulin formulations were stored at 8°C and room temperature (22°C) for one to three months in closed vials. All the formulations containing excipients in phosphate buffered saline appeared stable by physical examination and showed no sign of aggregation or change in colouration during storage. Although further investigation showed they appeared slightly cloudy after 3 months. However, in the case of insulin dissolved in HCl, at 8°C the solutions in the absence of excipients, the formulations appeared clear. Nevertheless, after 1 month, discoloration was evident (Figure 4.3a) at 22°C temperature.

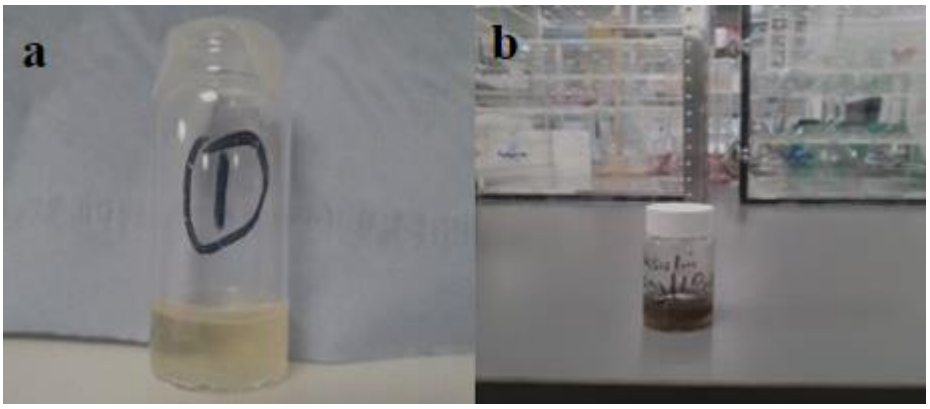


Figure 4.3(a) Native insulin stored at 22°C for a month, (b) 8°C for 3 months

1% w/v insulin in HCl stored at a temperature of 8°C was further observed after 3 months and a black colouration with lump likely to be identified as microbial growth (Figure 4.3b) was observed in the insulin formulation. Phosphate buffered saline appeared to be a better solvent

in storage stability study. Insulin can be hydrolysed during formulation, production or storage. Hydrolysis of amino acid peptide bonds occurs in both acidic and basic environments, compared to other amino acids, asparagine is more susceptible to hydrolysis and deamidation (Reubsaet et al., 1998). The asparagine amino acid, which is more prone to chemical instability, is relevant for modifying recombinant engineering to boost oral insulin absorption. The A21 asparagine in Glargine has been substituted by glycine to increase stability (Rosskamp and Park, 1999). Aside aggregation, temperature and pH must be considered throughout production and storage. High temperatures (usually above 50°C) can speed up protein breakdown and unfolding, However, low temperatures can weaken hydrogen bonding (Wang, 2005). Extremes of temperature should be avoided when synthesising peptide-based medications. In addition, while mixing excipients with insulin, the total pH must be considered (Privalov, 1990). Protein contact with the surface of their storage containers has the potential to be a major issue. The amphiphilic structure of protein molecules causes them to stick together, adhesion to a wide range of textures, as well as both their destabilization and failure of the protein structure (Felgner and Wilson, 1976) Protein adsorption on surfaces is a significant phenomenon that should be taken into account when formulating and choosing containers and closures for pharmaceutical products.

In parenteral administration, adsorption to a surface is a challenge. Adsorption of an inert protein, such as serum albumin, to saturate the container surface, or compounds that minimize surface interactions, such as surfactants, carbohydrates, or amino acids, may be used to alleviate this issue in these cases (Wang & Hanson, 1988; Law & Shih, 1999).

4.4.5 Thermal Stability and Aggregation of Insulin in Solution Form Investigated by Differential Scanning Calorimetry

The insulin formulation characterization began with a concentration (5mg of insulin dissolved in 1ml of 0.1 M HCl at pH 1.2, and 7mM phosphate buffer pH 7.6.) using the high sensitivity differential scanning calorimetry. As the experimentation progressed series of scan was carried out with a scan rate of 60min/hr from 20°C and 90°C. With a 1mg/ml, 3mg/ml, 5mg/ml concentrations, no reasonable transition was observed from the DSC. The insulin formulation was further adjusted to 10mg/ml.

Investigation was conducted on the thermal denaturation behaviour of human insulin in solution form. The thermal denaturation is partly reversible and complex as well as highly dependent on the association of insulin (Huus et al., 2005). The DSC results were fitted with different models according to the association state. The thermogram for insulin formulation with HCl fitted poorly to both a simple two-state transition and a non-two-state model due to the quality of thermogram obtained from the scans. Thermograms for insulin formulation with PBS was fitted excellently with a two-state model. Raw data of three independent replicates run with human insulin dissolved in phosphate buffer are shown in (Figure 4.4) representing the existence of two main endotherm peaks. Two distinct endothermic transition peaks were observed at Tm 76.36 and 87.29°C. Previous publications suggest, transition 1 and 2 represents dimer and hexamer denaturation (Huus et al., 2005). The thermal profiles for insulin were repeated and demonstrated a high precision of the high sensitivity DSC method during the investigation of large molecules. The thermograms observed during the experiment were model fitted using the Origin software.

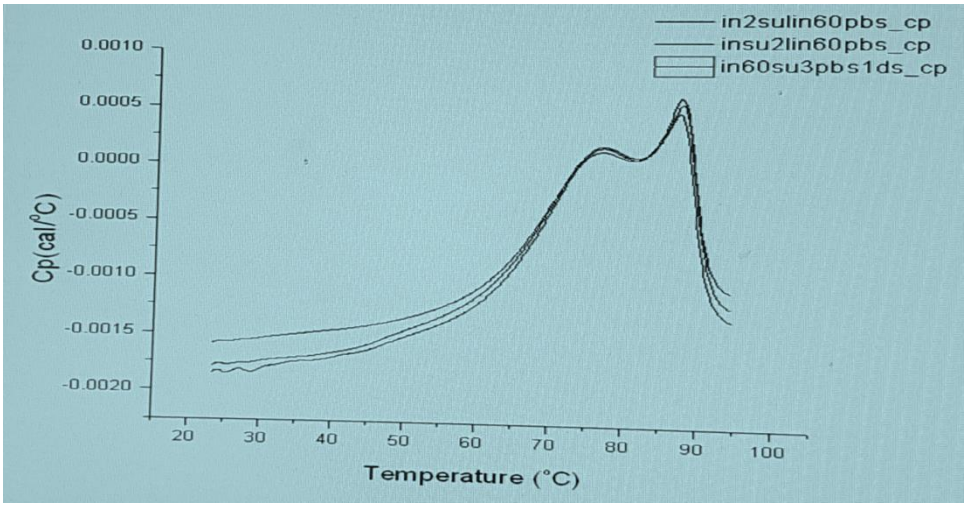


Figure 4.4 Independent replicates of a raw thermogram of 10mg/ml insulin in 7mM Phosphate buffered saline at pH 7.6 at a scan rate of 60°C/hr

Some of the endotherms observed were poorly fitted due to the small shoulder/peak observed for some formulations, however, a better fit was achieved when fitting two transitions with a pronounced peak (Figure 4.5).

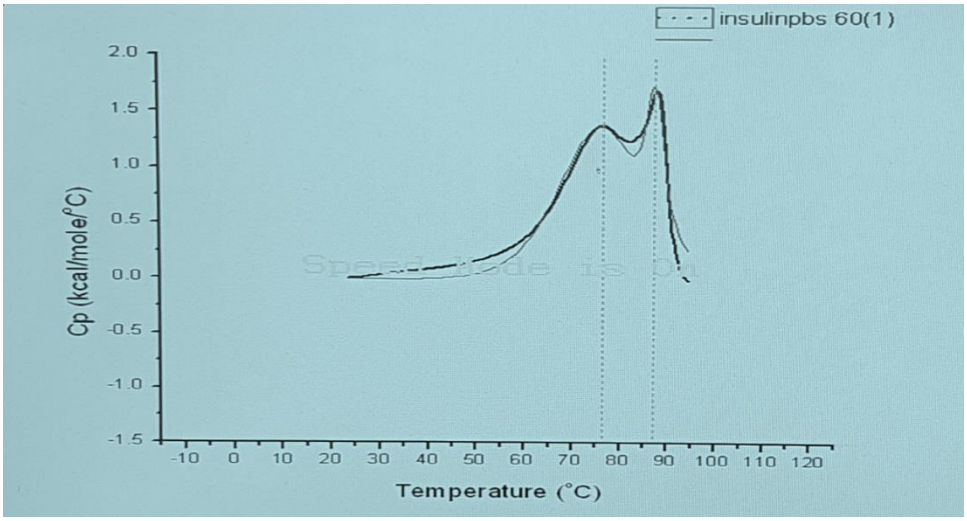


Figure 4.5 Showing a two-transition, two state unfolding model fitted thermogram of human insulin with the same conditions stated in Figure 4.4

The thermal denaturation mechanism must be understood in order to properly benefit from DSC for analysing changes in formulation compositions. To understand the dissociation and unfolding processes of insulin in different association states, DSC was used to investigate the

thermal stability of human insulin as a function of a 1:1% m/v ratio of excipient (beta cyclodextrin and pluronic F-127), with the same concentration of protein and excipients analysed. Table 4.3 outlines the denaturation temperature at two different scan rate (10 and 60°C), for the insulin formulations dissolved in phosphate buffered saline. From 70 °C, insulin, which is predominantly dimeric at room temperature, unfolded. Also, insulin concentration with phosphate buffered saline in the presence of excipients unfolded at about same temperature.

Table 4.3 The denaturation temperature calculated from the experimetal curve in phosphate buffered saline containing insulin.

FORMULATION	**DENATURATION TEMPERATURE**			
	60°C/hr Scan rate		**10 °C/hr scan rate**	
	Tm_1	Tm_2	Tm_1	Tm_2
1% w/v UNPROCESSED INSULIN	76.36	87.29	75.38	78.44
1% w/v INSULIN WITH BETA CYCLODEXTRIN	71.45	NT	74.06	NT
1%w/v INSULIN WITH BETA CYCLODEXTRIN AND PLURONIC	73.04	NT	69.40	NT
1 %w/v INSULIN WITH PLURONIC	28.66	71.45	27.52	81.2
MARKETED INSULIN	77.54	NT	77.45	NT

NT: No transition.

Marketed insulin: Lantus vial (Sanofi Aventis)

Some research state A biphasic thermal denaturation pattern of insulin might be generated by small quantities of zinc (Huus et al.,2005). A redistribution of zinc ions during the heating process causes biphasic denaturation, which resulted in two separate transitions with Tm's of 76.36 and 87 °C, corresponding to monomer/dimer and hexamer, respectively. The findings reveal that insulin's thermal stability is linked to its association state, and that the zinc hexamer can withstand far higher temperatures than the monomer.

Insulin's stability can be affected by concentration, ions and pH. Insulin monomers coexist alongside dimers and hexamers in solutions.

4.4.5.1 Effect of Excipients (Betacyclodextrin and Pluronic F-127) on Insulin Liquid Formulation

The DSC profile of native insulin dissolved in HCl and PBS (pH 1.2 0.1M; pH 7.6, 7mM) respectively with the addition of beta cyclodextrin and pluronic F-127 were analysed. The results from the DSC showed the thermal denaturaion temperature of insulin at 78.19°C in the absence of excipient. Figure 4.6 showed a slight shoulder endothermic curve with a massive exothermic peak.

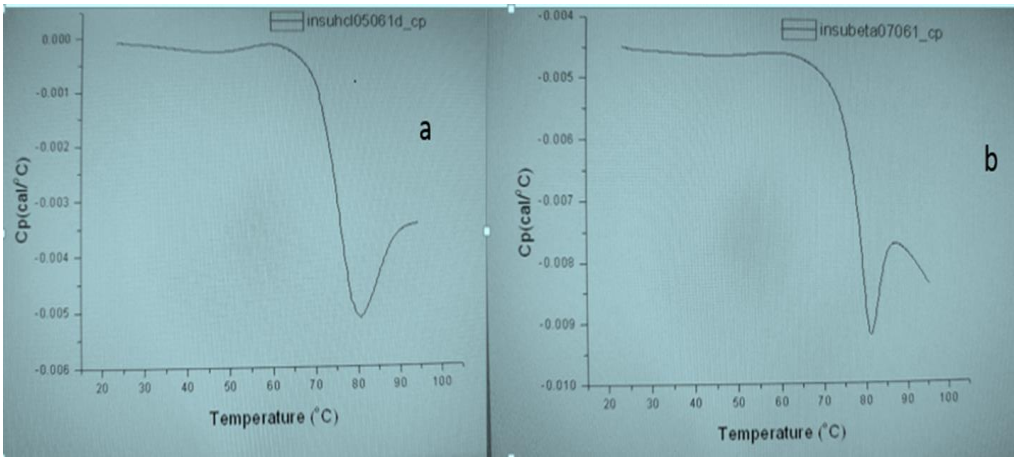


Figure 4.6: Illustration of a thermogram for (a)unprocessed insulin and (b)insulin with beta cyclodextrin insulin in HCl showing the aggregation temperature at 78°C and 79°C at 60°C/hr scan rate.

Insulin in the presence of beta cyclodextrin was depicted in Figure 4.6b with the existence of an exothermic peak as well. The presence of pluronic F-127 and a combination of both pluronic F-127 and beta cyclodextrin (Table 4.4) showed little or no effect on temperature for insulin formulation disolved in HCl.

Table 4.4: Showing aggregation temperature in T_m of insulin using High sensitivity differential scanning calorimetry (HSDSC) at 2 different scan rates (10 °C/ hr & 60 °C/ hr) respectively using HCl

FORMULATION	**SCAN RATE 60 °C/hr**	**SCAN RATE 10 °C/hr**
	T_m	**T_m**
1% w/v UNPROCESSED INSULIN	78.15	62.3
1:1% w/v INSULIN WITH BETA CYCLODEXTRIN	79.63	60.72
1:1:1%w/v INSULIN WITH BETA CYCLODEXTRIN AND PLURONIC F-127	78.31	60.58
1:1 %w/v INSULIN WITH PLURONIC F-127	78.18	60.82

However, addition of beta cyclodextrin alone exhibited a slight shift, with a temperature of 1.48°C towards a higher temperature. Interestingly formulation of insulin in the presence of pluronic F-127 containtion PBS (Figure 4.7) showed a remarkable event with slight shoulder exhibiting an endothermic peak at abput 70°C and a pronounced endothermic peak at denaturation temperature of 81.2°C. Pluronic F-127 appeared to have impacted on the stability of insulin using phosphate buffered saline while beta cyclodextrin had no effect on the stability of insulin.

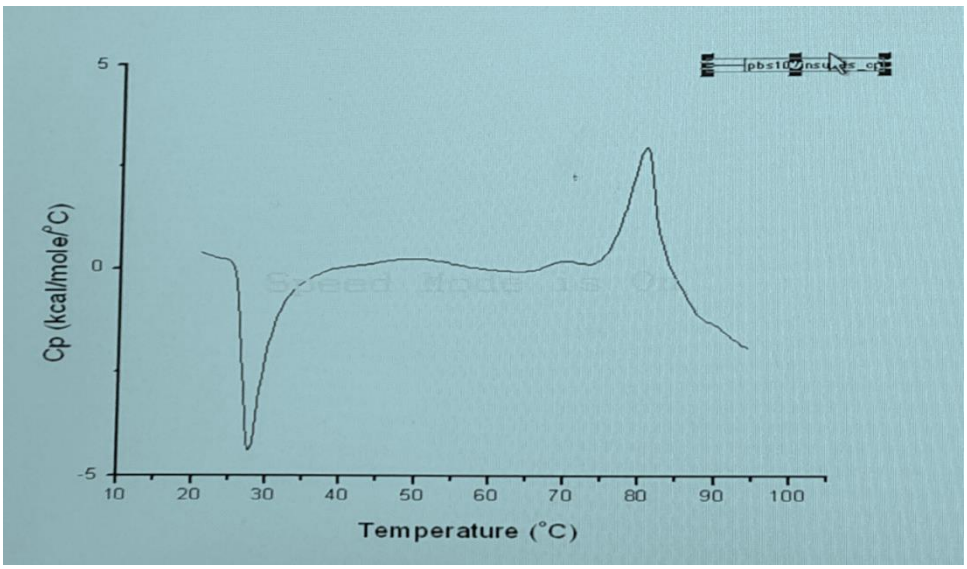


Figure 4.7 DSC scan representing a 1:1 %w/v insulin containing pluronic F-127 in Phosphate buffered saline pH 7.6.

A marketed insulin (Lantus) was purchased to analyse the thermal denaturation of the drug and compare with native insulin in the presence and absence of excipients. The active substance is insulin glargine, each 10ml vial contains 3.64mg. The ingredients include zinc chloride, metacresol, glycerol, sodium hydroxide. Hydrochloric acid and polysorbate 20. Figure 4.8. represents the DSC scan of prescribed insulin obtained and analysed with origin software. The thermogram depicted a single peak compared to unprocessed insulin in the absence of excipients.

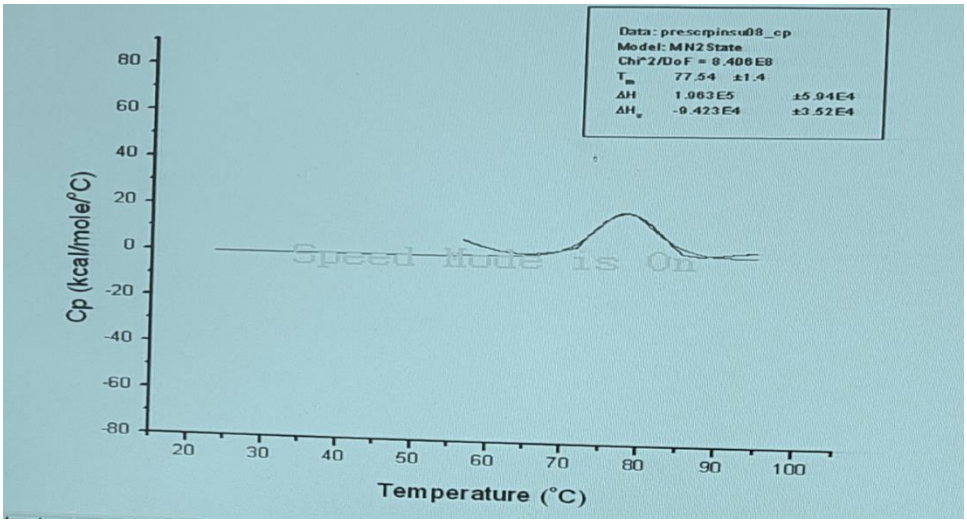


Figure 4.8 A DSC scan with a non- two state unfolding model fitted thermogram for marketed human insulin.

An increase in Tm (77°C) was observed compared to the insulin in the presence of beta cyclodextrin. This implies that addition of all the additives such as zinc chloride, metacresol, glycerol, sodium hydroxide and polysorbate 20 may have contributed to the increase in temperature. Insulin's mechanism is more complicated. Zinc binding results in the formation of stable insulin hexamers. The hexamer endotherm involves both dissociation and unfolding, whereas the zinc-free insulin endotherm does not exhibit dissociation behaviour. Thus, zinc binding causes both oligomerization and stabilisation, and the endotherms in biphasic denaturation are qualitatively different. There are no theoretical thermodynamic models that describe systems of this complexity (Huus et al., 2005). It would appear that zinc binding forces (complex binding) are more vulnerable to disruption by chemical denaturants than the forces that keep the protein folded together such as electrostatic forces, hydrogen bonding, hydrophobic interactions and van der Waals forces. The zinc coordination in the hexamer, on the other hand, has a much higher thermostability than the monomer fold (Huus, 2005).

Surfactant addition in formulation can minimize adsorption losses, for example, Tween 80 and pluronic F68 have showed to reduce calcitonin adsorption to a glass surface (Gidalevitz et al.,

1999) following research conducted by Gidalevitz et al using calcitonin which is a type of protein.

Self-association occurs in certain proteins, resulting in the formation of multimers, aggregates, and precipitates in extreme cases. Protein denaturation and non-covalent association via hydrophobic interfaces are thought to be the most common mechanisms of protein aggregation, insulin infusion therapy causes aggregation and gelling. Denaturation is commonly induced at gas-liquid, liquid-liquid, or container liquid interfaces, such as during the microencapsulation phase. Variations in pH, solvents, salts, and excipients may all play a role (Lougheed, 1980; shah, 1999; Jacob et al., 2006).

4.4.5.2 Effect of Scan rate on Insulin Liquid Formulation

The effect of varying scan rate of insulin formulation was investigated using microcal high sensitivity differential scanning calorimetry (DSC). A scan rate of (10 and 60 °C/hr) was used. The samples were loaded into the cells of the DSC and equilibrated for 15 minutes before the start of the experiment. The thermogram represents the excess heat capacity of the sample relative to the reference which is the buffer. When looking at the thermograms, the endotherms in the presence and absence of excipients appear the same for both scan rates but a decrease in aggregation temperature at about 62°C (Figure 4.9) was observed at a slower (10°C/hr) scan rate compared to about 80 ºC obtained for 60°C/hr scan rate (Figure 4.10).

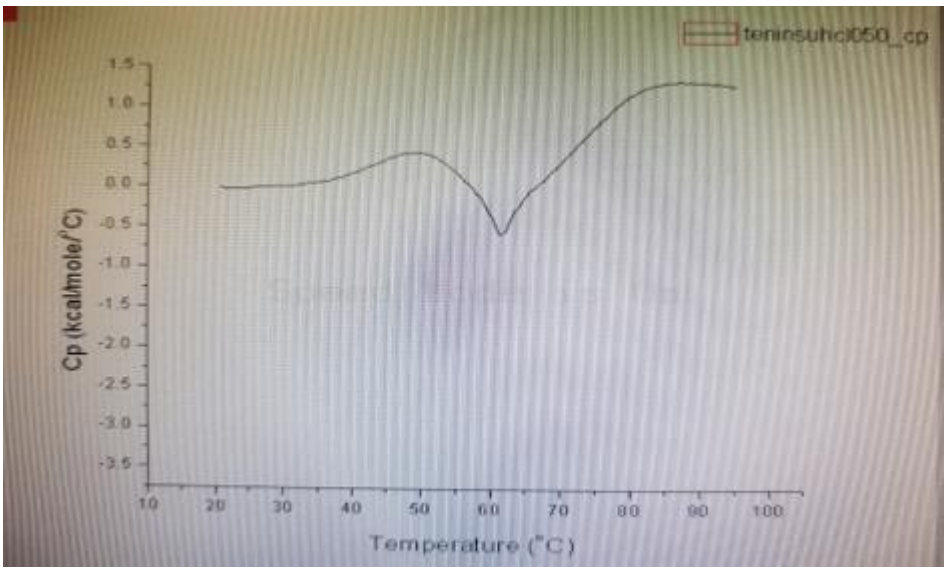


Figure 4.9. Thermogram of showing aggregated insulin in the following conditions: 10mg/ml protein, 0.1M HCl, pH 1.2 at 10°C scan rate

It was observed that a change in scan rate showed some effects on the shape and position of the (endothermic) transitions and influenced the exothermic peak as well, the higher the scan rate, the more shifted it is to a higher temperature and the broader the transition. It was further observed that from 50°C aggregations were too slow to be detected within the time frame of the experiment for insulin formulation with HCl; whereas from 60 °C , the effect was obvious and could be measured. A report by (Dzwolak et al., 2003) suggests the DSC tests for insulin yielded thermograms with a single endotherm transition followed by an exotherm at a low scan rate of 10°C/hr. This thermal denaturation profile is comparable to that which was produced with insulin in a report published by Dzwolak et al., 2003 at a pH of 1.2 with insulin dissolved in HCl. This is consistent with research on insulin denaturation at low pH. The results showed the endothermic unfolding occurring through an intermediate molten globule-like conformational state, following a native secondary structure unfolding. The protein aggregation that occurs as a result of the subsequent exothermic transition is irreversible. It was observed that at pH 7.4, the endothermic transition of human insulin is a non-two-state

process contrary to the result obtained for insulin in PBS at pH 7.6 which exhibited a two-state process (Figure 4.5).

Figure 4.10 displays a thermogram of insulin (10 mg/ml in HCl) at a 60°C/hr scan rate showing how aggregation accelerates rapidly with increasing temperature. The scan displayed a slight positive peak arising from an endothermic transition of about 60-70°C which progressed to an exothermic transition.

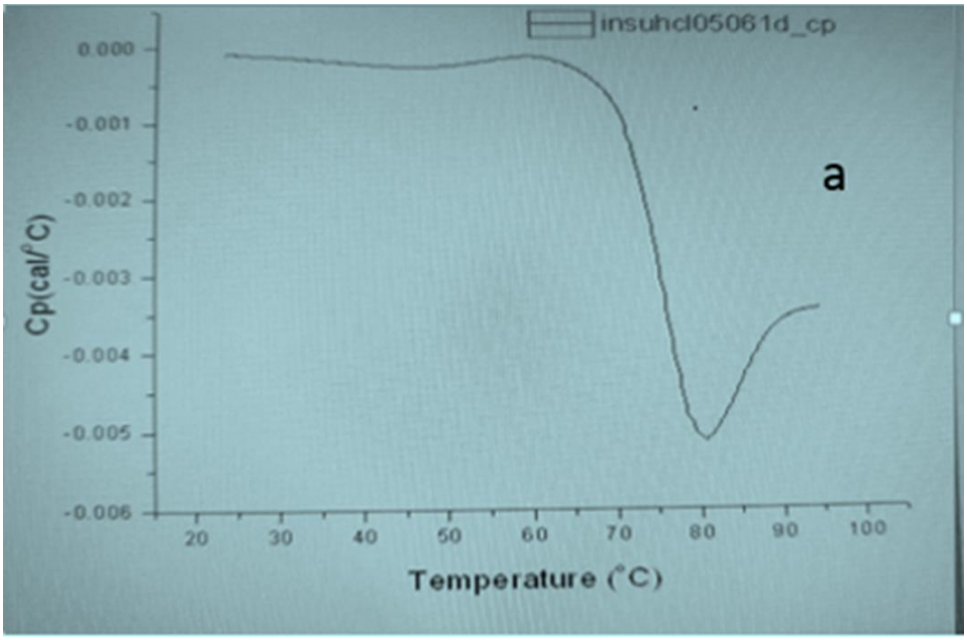


Figure 4.10. Raw thermogram showing aggregated insulin in the following conditions: 10mg/ml protein, 0.1M HCl, pH 1.2 at 60°C scan rate.

DSC scans revealed a single reversible endothermic transition with a Tm of 60 °C, followed by an exothermic peak. Report by Dzwolak et al (2003) confirms this study.

Dzwolak et al (2003) further report observation at a different pH and concentration, at a pH of 1.9 and a concentration of 20 mg/mL in HCl, differential scanning calorimetry (DSC) was utilised in order to research the aggregation of bovine insulin. Nielsen (2001) observes bovine insulin to be predominantly dimeric under these circumstances, the observation on the effect

of concentration on aggregation revealed that increased concentration increases both the endothermic and the exothermic transition. This research confirms this finding as the initial low concentration of (1mg/ml, 5mg/ml) intended for the study produced no transition until it was increased to 10mg/ml producing a significant transition. 1%; 2% and 3% of insulin in H_2O at pH 1.9 at 20°C/hr scan rate was also analysed in their study and the findings revealed that an increase in aggregation rate was connected with an increasingly exothermic character. Further, at 80°C and above, estimation of the effect of heat was feasible as the process apparently begins before the temperature scan is reached. It has been established that insulin aggregation is of an exothermic nature and demonstrate that the ΔCp of insulin aggregation is negative. This is in contrast to the unfolding process, which normally results in a positive ΔCp value (Cooper, 2005). The scan rate variation of the insulin samples showed a decreased Tm with a decreasing scan rate as indicated in (Table 4.5).

It is shown that organic solvents bind directly to proteins through hydrophobic interactions or modify the ionic strength within an aqueous media, resulting in the instability of protein molecules. In earlier research, it was demonstrated that the destabilising impact of organic solvents is mostly dependent on how the protein is integrated into the polymer (aqueous or solid form) and the organic solvent used for dissolution of the polymer.(Krishnamurthy & Lumpkin., 1998; Clealand & Jonees, 1996)

The thermal transition midpoint (Tm) from a DSC experiment is normally assumed to be in connection to the degradation process, based on the tendency of the protein to unfold, in stability screening for biopharmaceutical liquid formulations (Chiu et al., 2019). Several studies have found the relationship between the ranking of the physical stability of formulation measures as thermal stability by DSC and physical stability measured using other methods. Although this relationship is expected because agitation and DSC test, both relate to proteins folding stability.

Despite the fact that chemical degradation processes like deamidation, which occur at protein sites protected from unfolding in the native state, progress more quickly when the protein is unfolded, the relationship between long-term chemical stability and DSC results is not straightforward (Chiu et al., 2019). When it comes to protein aggregation, the effect of protein concentration may vary depending on the mechanism of aggregation and the experimental settings used. Brange et al reports bovine insulin fibril formation increases with an increase protein concentration from 0.75 to 5 percent during storage at pH 2.5 and 21°C. It was further stated that it takes less than 10 hours to produce the same number of fibrils at 5 percent and takes 15 days to be produced at 0.75 percent during storage at pH 2.5 and 21°C (Brange et al., 1997). During shaking, bovine insulin at 0.1 mg/ml at pH 7.4 aggregates more readily than bovine insulin at 0.6 mg/ml at pH 7.4. On the other hand, Sluzky et al (1992) suggests it may be due to a more complimentary production of insulin hexamers at 0.6 mg/ml, which are less vulnerable to hydrophobic surface-induced adsorption/aggregation than insulin monomers and are hence more stable (Sluzky et al., 1992). Chemical degradations can be influenced to a considerable extent by protein content in specific circumstances. In neutral solution at 37°C or 45°C, increasing insulin concentration causes a rise in the creation of covalent insulin oligomers and polymers (Brange et al., 1992a). Concentrated protein solutions, on the other hand, such as labile LDH, can be less susceptible to the protein aggregation and loss of action that might be caused by freezing than unconcentrated protein solutions (Carpenter et al., 1990, 1997). There are at least three (3) possibilities for this. The quantity of protein accumulated at the ice-water interface is limited, and as a result, the amount of interface-induced protein denaturation that can occur in concentrated protein solutions is limited. Protein unfolding may be inhibited by the steric repulsion of nearby protein molecules, which occurs secondarily (Allison et al., 1996). Protein–protein interactions can transform monomers into active and more stable dimers or multimers (Mozhaev and Martinek, 1984; Wang, 1999).

4.5 Conclusion

Excipients (beta cyclodextrin and pluronic F-127) and spray was employed in liquid formulations to minimise protein aggregation since protein aggregates can reduce biological activity, raise immunogenicity, or cause toxicity**.**

The spray drying of insulin using beta cyclodextrin and pluronic F-127 as excipients in this research showed some possible interaction. Beta cyclodextrin appeared to be a better excipient when comparing the percentage yield obtained. The conditions used for spray drying appeared unfavourable for formulation containing insulin.

Investigation on thermal stability showed pluronic F-127 appeared to have impacted on the stability of insulin using Phosphate buffered saline buffer while beta cyclodextrin had no effect on the stability of insulin. On the other hand, insulin in the presence of beta cyclodextrin in HCl prolonged the aggregation temperature by 1degree figure 4.6

Hence from this study, addition of pluronic F-127 to insulin formulation using phosphate buffer exhibited better stability and appeared to preserve the 3D structure of the protein compared to HCl that was used.

The utilisation of differential scanning calorimery approaches results in the production of analytical method for researching the aggregation of proteins. This is due to the fact that the occurrence of protein aggregation, occurs on the boundary between proteins as functioning protein, as polymers or biological molecules, and is determined by the link that exists between the structure of proteins and the hydration of those proteins. The discovery that aggregation of insulin takes place in two stages was made recently. The first stage involves the loosening of tertiary contacts and the endothermic unfolding of the insulin into a bulky intermediate. The second stage involves the irreversible, slow and exothermic aggregation of the insulin.

CHAPTER FIVE

5.0 CHARACTERIZATION OF ANTIBODY (IgG) AT DIFFERENT pHs IN THE PRESENCE AND ABSENCE OF BETA CYCLODEXRIN

5.1 Introduction

Novel excipients are needed to suppress protein aggregation, either as a replacement for existing excipients or as a supplement. The use of biotherapeutic has increased remarkably, making the quantitation of aggregate for large protein-based therapeutics of great concern due to their potential effect on efficacy and immunology (Hong et al., 2012) Many studies have shown that cyclodextrin is a promising class of excipients for preventing protein aggregation. They have shown to improve the thermal stability of liquid protein formulations, prevent surface-induced protein aggregation and be utilized as stabilizers during spray drying, also in the production of microparticles for therapeutic protein delivery (Serno et al., 2011). Parenteral administration in the form of an injection or infusion is most utilized due to the huge molecular weight and instability of biological macromolecules. As a result, it's no surprise that the majority of biopharmaceutical products available are in the form of dilute or concentrated solutions and suspensions (Bjelosevic et al., 2020).

The pharmaceutical industry is interested in the thermal unfolding profiles of monoclonal antibodies as they are useful for therapeutic targets. Aggregation is arguably the biggest challenge for the development of stable formulations and robust manufacturing processes of therapeutic proteins. In an ideal world, enhancing both colloidal and conformational stability would be advantageous also. Creating a stable formulation is one thing, but optimising one is another because the variables may jeopardise the other. When it comes to formulating mAbs, there are few options to consider when monitoring protein aggregation. DSC is a tool used to assess temperature stability and viability of monoclonal antibody (Schon and Freire, 2021),

the speed of size exclusion chromatography and its repeatability have made SEC a popular choice for routine and verified analysis of biological samples (Brange et al., 1992; Yu et al., 2008). DLS and optical spectroscopic techniques are also widely used to characterize aggregation and size to measure the monomer, dimer, trimer and the higher-order oligomer (Barnard et al., 2011). High concentration API formulations are used for many modern mAb therapeutics (Maruyama et al., 2022; Bansal et al., 2020; Douglass et al., 2015; Startzel et al., 2015; Neergaard et al.,2014) and some older immunoglobulin, however this study will focus on low concentrations.

This chapter reports the effect of pH, Beta cyclodextrin and concentration on the stability of IgG formulations. In this chapter, high sensitivity differential calorimetry (HSDSC), dynamic light scattering (DLS) and high performance liquid chromatography (HPLC) were used to study the thermal stability and aggregation of two sources of therapeutic protein (human IgG and bovine IgG) at low concentrations.

5.2 Aim

The instability and aggregation propensity of this protein drug poses some serious challenges to the pharmaceutical industry,.This brings us to the aim of this chapter which is to study the influence of temperature on monoclonal antibodies in the presence and absence of a stabilizing excipient (beta cyclodextrin).

5.3 Objectives

1. The thermal stability of IgG in solution using high sensitivity differential scanning calorimetry (HSDSC) and dynamic light scattering (DLS) will be investigated, and the effect of the presence and absence of additives on thermal denaturation and aggregation will be assessed.

2. The influence of the source of IgG on the denaturation temperature will be observed.
3. Investigation on the effect of pH on thermal analysis and aggregation on monoclonal antibody using Dynamic light scattering (DLS) and Size exclusion high performance liquid chromatography (SEC-HPLC).

5.4 Results and Discussion

5.4.1 Thermal Denaturation of Monoclonal Antibody Immunoglobulin (IgG).

Thermal denaturation of IgG molecule was analysed using High sensitivity DSC melting curve at a scan rate of 60°C/hr. The IgG formulations at a concentration (1mg/ml and 2.4mg/ml) was prepared and filled in the cell of the DSC equipment. The samples were heated at a temperature between 20-90°C. The raw data of the human IgG (1 mg/ml) in a 10-mM sodium citrate, 140mM NaCl buffer, pH 5.5 in (Figure 5.1) reports an endotherm at about 60°C and a rapid exothermic reaction. The samples were reportedly cloudy after heating as shown in (Figure 5.2) indicating a level of precipitation. This necessitated the DSC system being washed with nitric acid to remove precipitated protein in the system and ensure system accuracy to continue with other formulation assessment. The thermal scans for the IgG were repeated. The origin 7.0 software model was attempted in the fittings of the endotherm of the scan results. Several fittings were attempted before obtaining a better fit. Some of the high temperature endotherms could not be model fitted due to unknown errors. The DSC curve showed a slight transition at a temperature denaturation of 68.08°C with an aggregation peak at temperature 78.54°C. The transition represents the denaturation of the F_c and F_{ab} domain. Each domain can be seen individually as they thermally unfold using DSC (Vermeer et al.,1998; Garidel et al., 2020)

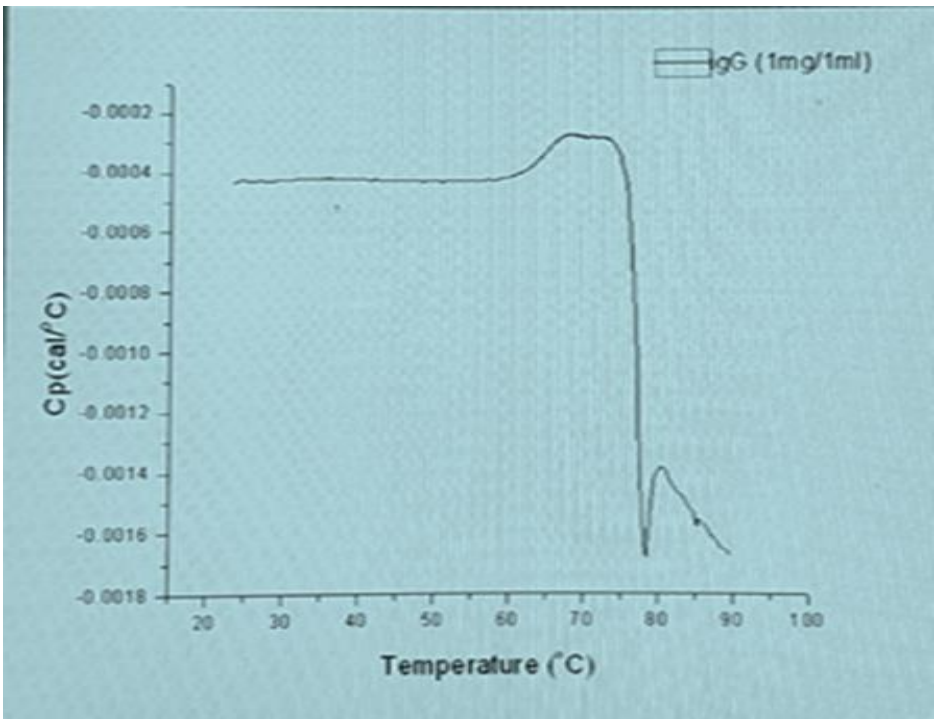


Figure 5.1 DSC thermogram of human IgG (1mg/ml) in a 10-mM sodium citrate, 140mM NaCl buffer, pH 5.5.

Denaturation of the domains were observed with an onset melting temperature at around 60°C and the denaturation of immunoglobin G becomes notably irreversible at over 65°C (Akazawa-Ogawa et al., 2018; Indyk et al., 2008; Mainer et al., 1999). The findings from these researchers confirm the event in this study.

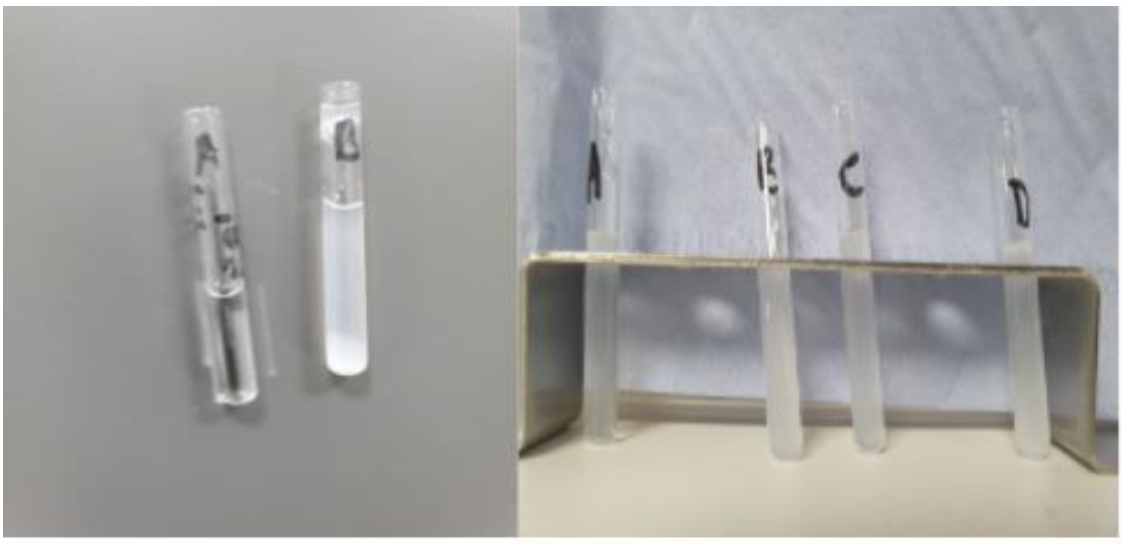
The peaks observed represent F(ab) and F(c) fragments of the IgG molecule. Fab fragment appears to be the most reactive to heat, on the other hand, Fc fragment is sensitive to decreasing pH. The transitions were independent and an irreversible aggregation was immediately followed. The unfolding is the step in the overall denaturation process that determines the rate at which it occurs. (Wang, 1999). In addition, the denaturation process determines the structure of the aggregates generated and IgG molecules become bound in aggregates before they can be totally denatured. The human IgG showed some slight endotherm reflecting events of unfolding as expected from large multidomain structures, given the complexity and size of antibodies, interesting, thermal denaturation did not start below 55-60°C. This is consistent with previous study (Buchner et al., 1991). After being subjected to heat treatment for several

minutes at 90°C, IgG virtually totally loses its antigen-binding function. Following the different type of immunoglobulin, studies conducted previously showed that each immunoglobulin domain begins to unfold at varying temperature, for example in IgG, the CH3 domain unfolds at a higher temperature than its CH2 domain. The immunoglobulin CH2 domain serves as a convenient model system for studying the folding of all proteins. It comprises of two β-sheet which form a Greek-key β-barrel, stabilised by an internal disulphide bridge in the hydrophobic core. Various antibodies crystal structures indicate that the two heavy chains CH2 domains interact with their sugar moieties and create a homodimer (Feige et al., 2004). Thus, antibody has a mixture of the unfolded and folded structures at a certain temperature. It is possible that the presence of both unfolded and folded domains in a single polypeptide chain will increase the tendency to aggregate, which will result in the inactivation of antibodies (Akazawa-Ogawa et al., 2018). As denaturation of IgG sometimes caused by heat is usually irreversible, precise analysis of DSC data is impossible. However, when the experimental setting is identical, the results allow us to examine the relative stability of domains in a single IgG and those in distinct IgGs (Akazawa-Ogawa et al., 2018).

In a way, the fact that the constant domain of isolated Fab denatures more rapidly than the variable domain supports the hypothesis that multi-domain proteins can be represented by the denaturation of their constituent domains, as reported by Goto and colleagues (1988). A multi-domain protein's domains are supposed to denature independently, however it's likely that multiple denaturation pathways exist, with varying domains being impacted in nonidentical order based on the protein. Depending on the circumstances, one or the other may be preferred. (Vermeer and Norde, 2000).

To determine the thermodynamic parameters of each transition, it is necessary that the domain transitions are adequately separated along temperature. Some immunoglobulins show many transitions in their DSC thermograms, but the overlap between the peaks is too strong to allow for a detailed thermodynamic characterization of the individual transitions (Tischenko et al.,

1982; Shimba et al., 1995; Arnoldus et al., 2000). This software can rapidly deconstruct the transition envelope to predict the transitions in each domain even when these transitions heavily overlap (Vlasov et al.,1996). If an active drug material is held at a refrigerated temperature of 2 to 8°C for the length of formulation development, it usually does not degrade. As a result, temperature-induced stress studies are frequently employed in order to obtain enough degradation that can be captured by characterization methods. The results of the temperature-induced investigation are thought to be relevant to the rate of degradation during storage situation (Zheng et al., 2015). The protein IgG is classified as a multi-domain protein, the appearance of two transition peaks could indicate the existence of at least two domains, or groups of domains, that denature under different conditions. Vermeer et al (1998) and Oi et al (1984) reported the thermogram of IgG of isotype 1, showing only a single transition peak. It was observed that thermograms of IgG obtained in the study displayed one endothermic peak before aggregation. It was further stated that the difference between the two IgG isotopes may be as a result of increased flexibility of the hinge region of the IgG used in the investigation. This has also been proposed by Garidel et al (2020).



(a) **(b)**

Figure 5.2 (a) human IgG solution prepared before and after heating. (b) different formulations of aggregated human IgG after heating in the presence and absence of excipient

The Figure 5.2 (b) shows the outcome of (a) native IgG (1mg), (b) native IgG (2.4mg) (c)IgG+beta cyclodextrin (1mg/ml, 1:1 concentration (same concentration of IgG and betacyclodextrin)) and (d) IgG+beta cyclodextrin (2.4 mg/ml, 1:1 concentration. All the samples after heating appeared cloudy and aggregated due to protein coagulation.

5.4.2 Effect of Solution Conditions on the Thermal Denaturation and Aggregation of IgG

Characterization using various techniques to study the effect of pH on the thermal stability and aggregation of IgG was observed at a low concentration of (1mg/ml; 2.4mg/ml). IgG in 10-mM sodium citrate buffer with 140mM NaCl, pH 5.5, 7.4 respectively was used. The samples were prepared and analyzed with DSC, DLS, and SEC-HPLC techniques to investigate the thermal stability and aggregation of Human Immunoglobulin. A water ACQUITY UPLC Protein BEH SEC column 200 A (1.7Um, 4.6 x 300 mm), 0.2M potassium phosphate buffer containing 0.25M potassium chloride at a flow rate of 0.3 ml/min was used for SEC analysis. The thermal stability was studied during heating from 20-90 °C and after heating. The effect of the solution condition was based on the pH (5.5 and 7.4) used for the antibody characterization.

Table 5.1 Midpoint temperature (T_m) and aggregation T_{agg} of human igG, n=3

Formulation	**Human IgG**	
Concentration	**1mg**	**2.4mg**
T_m(°C) pH 5.5	68.08±2.5	67.47±4.6
T_{agg} (°C)	78.54	75.00

T_m(°C) pH 7.4	71.51± 1.4	70.66+0.35
T_{agg} (°C)	82.00	76

Investigation of the pH dependence on thermal stability and aggregation performed by DSC was observed at two different pH (5.5 and 7.4), the scans were carried out at 60^{o}C /hr, the Tm(s) followed the same general trends, as shown in the thermogram figures, see appendices xi. The thermal transitional derived from the formulations at both pH were similar and comparable, as well as those for Tm values. Formulations containing 10 mM citrate buffer NaCl at pH 7.4 for Human IgG showed little or no difference in Tm value compared to that of pH 5.5 considering the standard deviation values overlapping between the samples. The citrate buffer formulations at pH 5.5 also showed no difference in Tm values ranging from 67.47 ^{o}C to 68.68 ^{o}C compared to 70.66 ^{o}C to 71.51^{o}C of pH 7.4 comparing with the standard deviation figures (Table 5.1).

The monoclonal antibody (IgG) formulations were further characterised using dynamic light scattering instrument to analyse the particle size of the samples. During the particle size analysis, the formulations analysed were not seen to be different at both pH 5.5 & 7.4 respectively (Table 5.2 and 5.3). The particle size at pH 7.4 was observed to be between 201.2 nm to 315.6 nm compared to formulations performed at pH 5.5 with particle size ranging from 188 nm to 1862 nm. The heated formulations from the DSC were also analyzed after heating, the samples were aggregated and formed white precipitate as earlier stated. DLS analysis showed the size of the particles increased drastically after heating. There was evident increase in size in the formulation ranging from 188 nm to 8290 nm for pH 5.5 and 201 nm to 5982 nm for pH 7.4 respectively. This could appear to be degradation linked to aggregation of the formulations confirming the aggregation Tm(s) observed in the DSC scans. The large particle size shown in Table 5.2 is as a result of heating carried out on IgG formulations from 20 to 90 ^{o}C. This analysis was performed before and after heating. The increase in temperature

produced a large formation of assemblies from 188 nm at 20 °C before heating with DSC to 4607 nm after heating at 90 °C as the samples withdrawn from the DSC after heating and visual inspection evidently showed large particles.

Table 5.2 Showing the particle size (d.nm) and PDI in mean standard deviation for IgG formulation in the presence and absence beta cyclodextrin at pH 5.5 before and after heating. n=3

Formulation	HIgG		HIgG+beta		HIgG		HIgG+beta	
Conc	1%w/v	heat	1:1%w/v	heat	2.4%w/v	heat	1:1%w/v	heat
Size (d.nm)	188.9±124.8	4607±2202	622.0±215.9	4476±814.3	1670±486.4	8290±1858	1862±36.77	3019±78.55
PDI	0.508±0.084	0.857±0.284	0.688±0.063	0.901±0.243	0.997±0.008	0.829±0.297	1.000±.0.00	0.411±0.079

Table 5.3 showing the particle size(d.nm) and PDI in mean and standard deviation for IgG formulation in the presence and absence beta cyclodextrin at pH 7.4 before and after heating n=3

Formulation	HIgG		HIgG+beta		HIgG		HIgG+beta	
Conc	1%w/v	heat	1:1%w/v	heat	2.4%w/v	heat	1:1%w/v	heat
Size (d.nm)	201.2 ±87.66	3007±988.6	166.9 ±46.48	5982±161.7	625.0±23.86	2038±482.1	315.6±163.5	2793±39.29
PDI	0.526±0.054	0.662±0.377	0.266±0.086	0.332±0.068	0.200±0.057	0.775±0.190	0.514±0.191	0.258±0.138

The data from DLS showed that when the human IgG was heated, the change observed was a disturbance of the tertiary structure. When temperature continued to rise, there was evidence of aggregation, and the process of aggregation resulted in the loss of the native alpha-helix and an increased non-native intermolecular beta sheet (Zhou et al., 2015). Therefore, it is essential to keep in mind that the PDI value is a measurement of homogeneous size distribution, and it does not automatically imply that the whole sample is highly aggregated. This understanding will assist in the interpretation of these data. The PDI value for the 1 mg/mL sample after completing the complete heat /cool cycle is less than 1, which is the highest possible value; however, there are large peaks in the particle size distribution, with 99.9 percent of the mass accounted for by the monomer particle population with a diameter of 188 nm, and above. Only a few percent of the sample mass is present as an aggregate. The 2.4 mg/mL sample at pH 5.5, on the other hand, tells a different tale, in that while the PDI value is only 0.9-1 before heating, in the presence and absence of beta cyclodextrin, big aggregates account for about 100 percent

of the total sample weight. When looking at the particle size distribution, there are substantial populations that indicate serious aggregation has occurred from 1670nm to 8290nm diameter. It is not possible to fit the DLS correlogram with validity after reaching the temperature because the number variations caused by huge aggregates distorted the curve (Figure not presented).

Under physiological conditions, the protein backbone is very stable; however, certain sites may become more susceptible to fragmentation due to factors such as the amino acid sequence (the presence of specific side chains that may facilitate cleavage), the flexibility of the local structure, solvent conditions (pH, temperature), and the presence of metals (Vlasak and Ionescu, 2011). Monitoring the process of modifying monoclonal antibodies (mAbs) is an essential quality feature that needs to be done in order to assess the intergrity and purity of proteins.. Fragmentation during synthesis in protein cells, is influenced during the process of purification, and accumulates as it is being stored or while it is circulating in the blood. In general, the pattern of fragmentation of a monoclonal antibody acts as a fingerprint of both the manufacturing process and the consistency of its stability. Because of this, it is an essential aspect in assessing whether or not materials produced in different locations are comparable to one another (Vlasak and Ionescu, 2011). Size exclusion HPLC was further used to characterize the IgG formulations, with detection at 214nm, 220nm and 280nm. Instability was forced with a high temperature of about (60 and 75°C). Size exclusion chromatography (SEC) was used to observe protein aggregation for all the prepared and heated samples (forced degradation), This was carried out in line with findings in the experiment conducted previously on DSC where samples were heated at 20-90°C for an hour. A loss of signal linearity was observed at 2500 mAU approximately, at a range between 200 and 2500 mAU, detection of monomer was optimal maintaining signals above LOD for peaks like oligomer with low intensity (Molloy et al., 2015). The chromatogram showed a separation of the light and heavy chain achieved with BEC 200 column. It is important to mention that this analysis was carried

out under specific conditions, one flow rate and mobile phase. The results obtained during analysis indicated a percentage loss in the SEC monomeric peak area (Fig 5.3) for 1mg IgG at pH 5.5 compared to pH 7.4. The retention time at pH 5.5 for 1mg IgG was observed at 7.994 mins and 7.980 min for pH 7.4. See appendices xii showing the specificity of the different peaks where IgG peaks were observed at about 7 mins and buffer at 12 mins retention time.

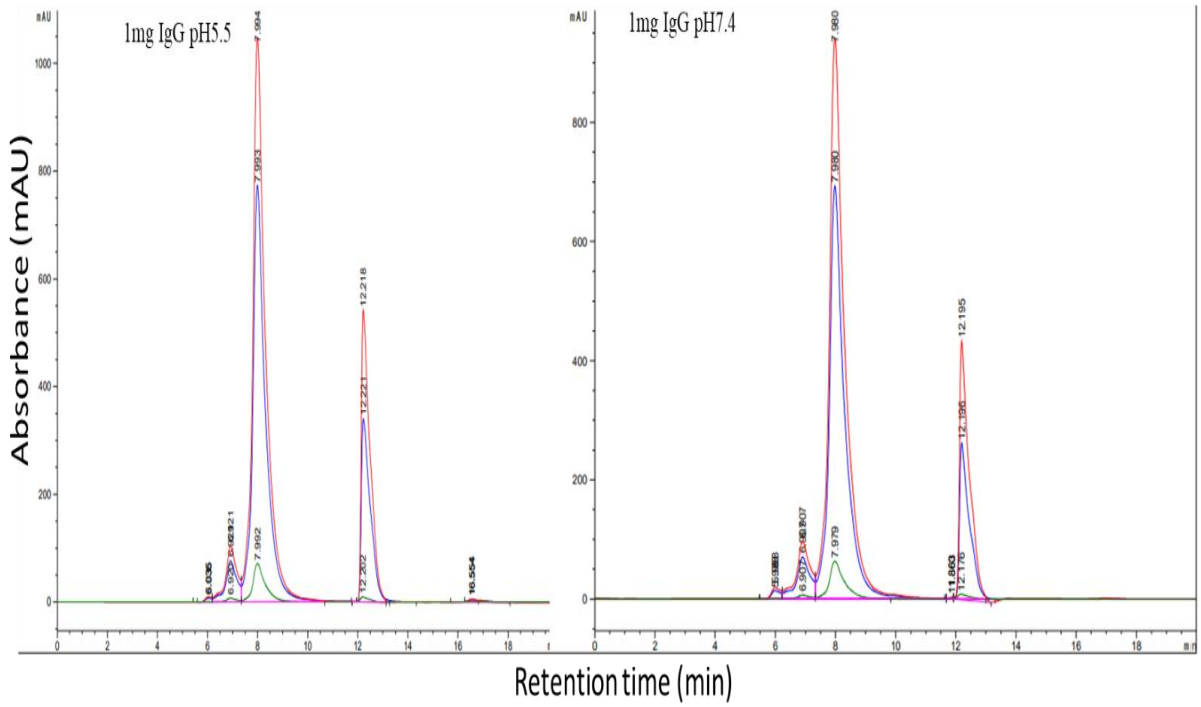


Figure 5.3 Comparison of peak area and retention time of IgG at pH 5.5 and pH 7.4 heated at 60°C. n=3

For samples heated at 60°C, there was a prominent peak displayed however a major change occurred for samples heated at 75°C. The retention time for the sample heated at 60°C was observed at 7.9 min whereas no meaningful peak indicating the presence of IgG except a buffer large peaks at 13.198 mins observed for the sample heated at 75°C (Figure 5.4). This could be an indication that aggregation had taken place. Temperature should usually have no effect on retention in SEC separations but influencing the protein structure, as well as mobile phase viscosity and analyte diffusivity, temperature can have some indirect influence on retention (Hong et al., 2012).

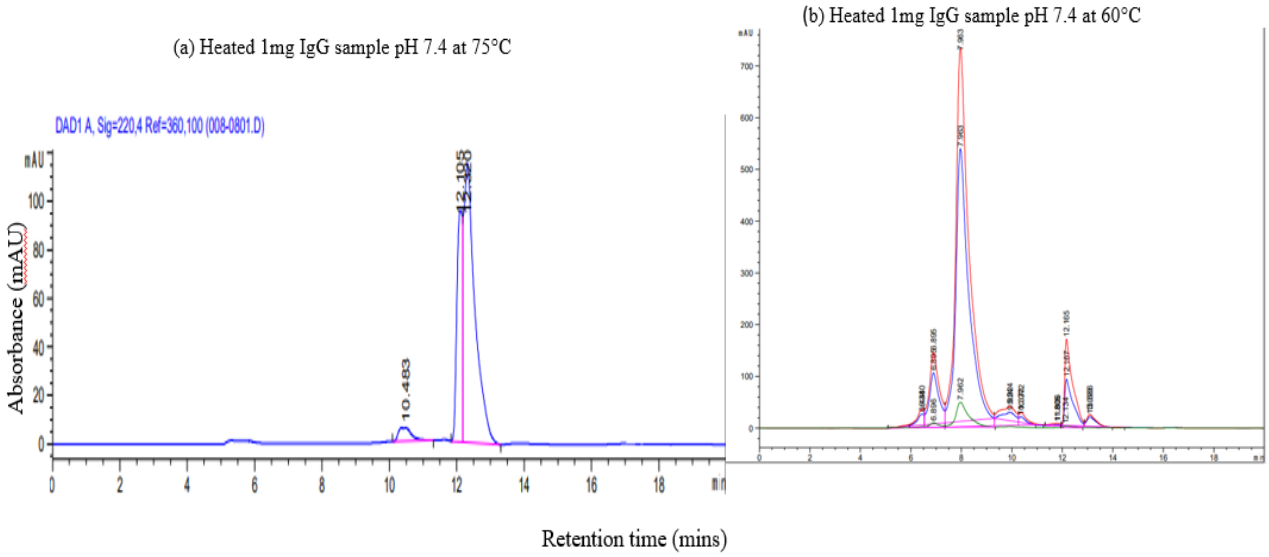


Figure 5.4 Showing retention time of 1%w/v 1mg/ml heated samples at temperature 60 and 75°C at pH 7.4. n=3

The pH of a solution influences the protein stability physically and chemically, making the choice of a buffer for stabilising the formulation crucial. The most frequently encountered are His, citrate, acetate or phosphate buffer, all of which fall within the range of pH 3–9. When several buffer options are available for a particular target pH, it is common to find differences between specific buffer ions in terms of their influence on specific degradative pathways or the rate at which they degrade. In the example of RMP-7 (a drug named lobradimil which helps other drugs reach the brain), a nine amino acid peptide, the utilisation of an acetate and citrate buffer to achieve the desired pH of 4 was employed. It was ascertained that the use of citrate buffer resulted in peptide breakdown occurring more quickly than the use of acetate buffer. This demonstrates the unique effect of buffer ions on formulation. In order to prevent the effect from being predicted or generalised to other molecules, necessary understanding of the features of the unique medication molecule is important (Jain et al., 2019). The protonation and deprotonation actions of particular amino acids (often asparagine, glutamine, and histidine

at acidic pH values) demonstrating pKa difference in the native and denatured forms is what causes dependency of pH of protein stability (Karp et al., 2007; Schon et al., 2021). For the CH2 domain, the pH dependency of the Gibbs energy can be stated in terms of the apparent number of protons received and released, as well as the pH halfway of the process of protonation and deprotonation. The shift in pH that occurs with Tm is caused by the fact that ΔG also known as change in entropy, is the quantitative measure of a favorable given reaction under certain condions, is pH dependent (Schon et al., 2021)

5.4.3 Effect of Protein Concentration on IgG Thermal Stability

To observe the effect of protein concentration on human IgG thermal stability, 1 and 2.4mg/ml of immunoglobulin was analysed at pH 5.5 and 7.4. The thermogram of the immunoglobulin showed transitions exhibiting the denaturation process for the IgG concentrations explored. The transitions showed different sensitivity to temperature based on the concentrations applied. The rate of aggregation occurred faster at higher concentration of 2.4mg (Figure 5.5) whereas the relatively lower concentration of 1mg occurred at a higher temperature.

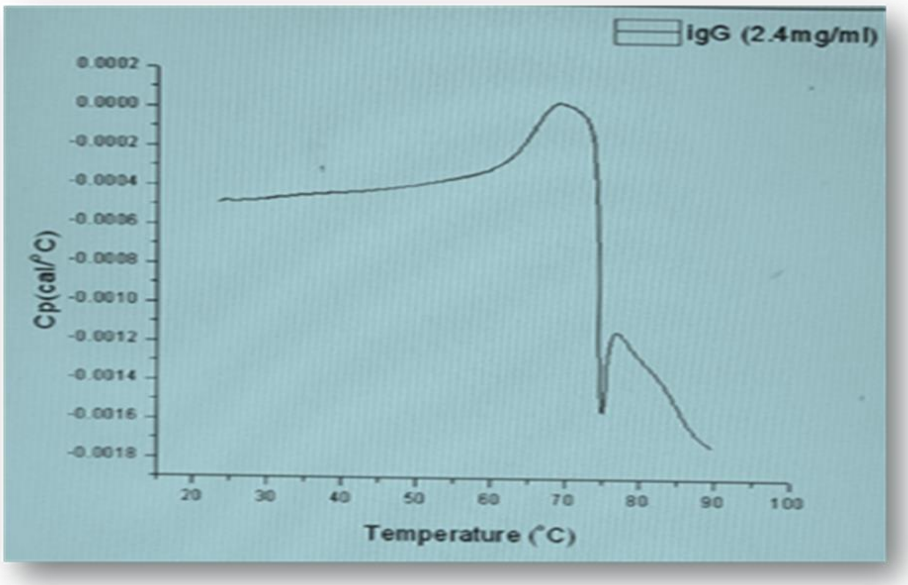


Figure 5.5 DSC thermogram of human IgG (2.4 mg/ml) in a 10-mM sodium citrate, 140mM NaCl buffer, pH 5.5.

When compared to Figure 5.1, the aggregation temperature was observed at 79°C for 1mg/ml IgG and 75°C for 2.4mg IgG. The IgG concentration of 1mg/ml showed a slight shoulder beside a broad endotherm with a T_m of 68°C compared to the IgG concentration of 2.4mg/ml (Figure 5.5) showing a slightly similar endotherm. At different concentrations, they both exhibited exotherms at different aggregation temperatures while the T_m for both concentrations showed minimal effect as a difference in the width of the peaks was observed. Intermolecular interactions are known to be responsible for changes in thermal profiles caused by different concentrations, Paborji et al., 1994; Douglass et al., 2015 reported various concentrations ranging from 1-10mg/ml of antibody showing minimal influence on the thermal stability of mouse-human IgG in citrate buffer at pH of 5.5 exposed under a temperature of 60°C and suggested that concentration effects may be influential only at higher temperatures above the onset of denaturation at approximately 60°C as deduced from calorimetric data. This study is in agreement as both concentrations showed an endothermic effect and an exothermic effect afterwards. Protein aggregation is generally dependent on concentration. The mean-field lattice model predicts that protein will aggregate or precipitate at sufficiently high temperature (Fields et al., 1992, Wang 1999). According to Ruddon and Bedows's (1997) research, a rising protein concentration that is greater than 0.02 mg/ml has the potential to facilitate the formation of protein aggregates. The protein aggregates size may also rise with an increase in the concentration of the respective protein, such as b-lactoglobulin as reported by Roefs and De Kruif, 1994. Data obtained from DLS analysis (Table 5.2) suggests an increase in concentration impacted the particle size of the formulations as an increase from 188nm to1670nm for pH 5.5 and 201nm to 625nm for pH 7.4 formulation was observed. The increase in size observed after heating is attributed to aggregation as well. A faster retention (7.6min)

time was observed for 2.4mg IgG in SEC-HPLC study compared to 0.3 mins delay observed for 1mg IgG (7.9min) oberved in Figure 5.6.

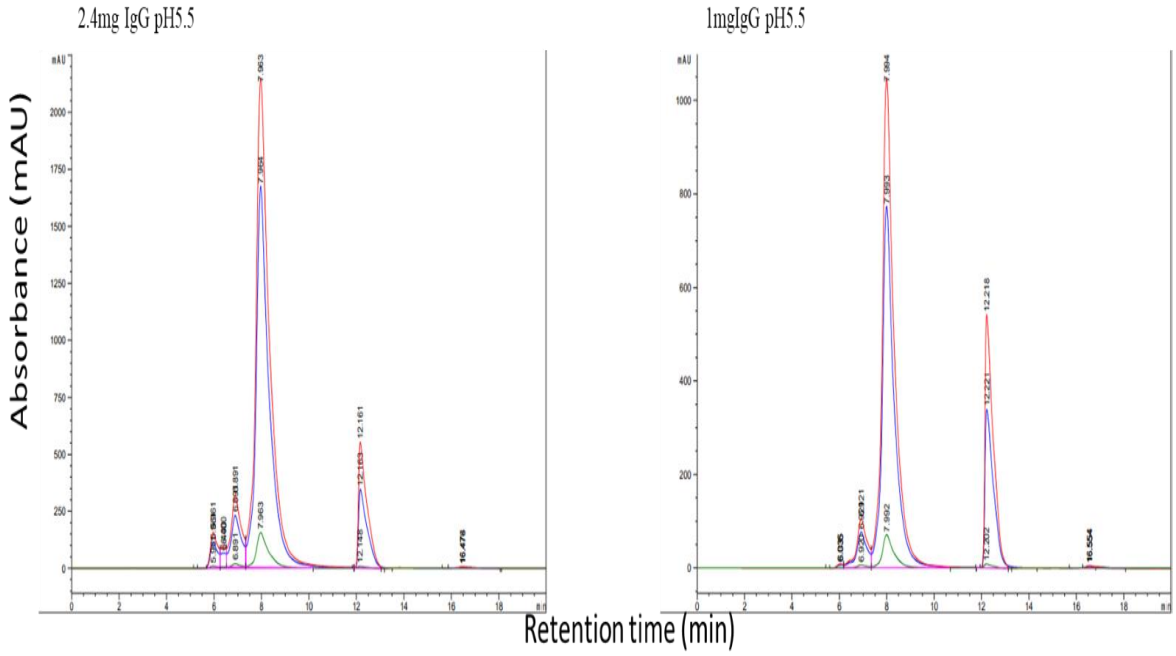


Figure 5.6 Comparison of peak area and retention time for 1mg and 2.4mg concentration at pH 5. n=3

For 1mg of IgG, there was seen to be a drop in percentage in SEC monomeric peak area (71%) while 2.4mg had a higher percentage area of 77%. The mechanism of aggregation and the experimental settings both play a role in determining how the concentration of a protein affects its ability to form aggregates. Report suggests in scientific literature that formulations with high concentrations of protein aggregate is due to the high viscosity of the formulations. However, it is important to keep in mind that monoclonal antibodies (mAbs) formulations are typically given in low concentrations (less than 10 mg/mL) in large volumes. On the other hand, the creation of protein formulation in high concentration (between 50 and 150 mg/mL for monoclonal antibodies) attempted to promote compliance by lengthening the time between administrations (Pharm and Meng 2020).

5.4.4 The Effect of Beta cyclodextrin on the Temperature Transition of IgG

There is a wealth of evidence in the scientific literature demonstrating how a variety of excipients each provide their own unique ability to prevent protein aggregate formation (Ohtake et al., 2011). IgG's solution thermal stability and aggregation were investigated in the presence of beta cyclodextrin. In this study, it is documented how beta cyclodextrin influence thermal stability and aggregation using DSC, DLS and HPLC. In determining the effect of beta cyclodextrin, Figure 5.7 represents the raw thermogram in the presence and absence of beta cyclodextrin, the presentation of the thermograms were different as 1:1(1mg/ml) IgG:beta cyclodextrin showed a slight shoulder with a peak extension before aggregation compared to the higher concentration with a more definite shaped peak.

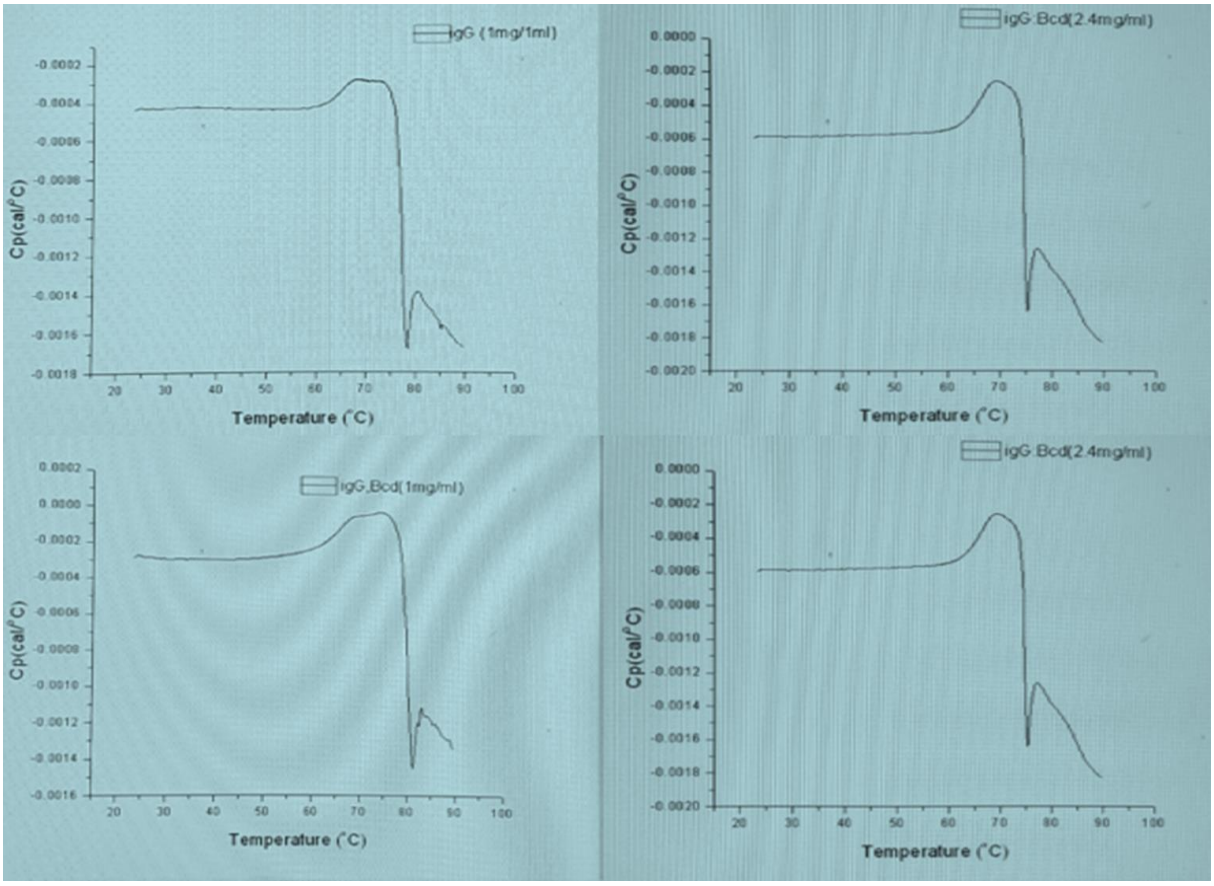


Figure 5.7 Raw DSC thermogram of human IgG (1mg/ml,2.4 mg/ml) with and without beta cyclodextrin in a 10-mM sodium citrate, 140mM NaCl buffer, pH 5.5.

Table 5.4 outlines the T_m (s) obtained from the thermogram of IgG in the presence of beta cyclodextrin. The presence of 1%w/v beta cyclodextrin in 1mg/ml IgG at pH 5.5 showed no positive effect on the denaturation temperature at 68.68°C compared to 68.08°C in the absence of beta cyclodextrin considering the standard deviation corresponding to mean value. Using an increased concentration of 2.4mg/ml, the effect of beta cyclodextrin on IgG was 67.90°C compared to 67.47°C in the absence of beta cyclodextrin.

Table 5.4 Midpoint temperature (Tm) and aggregation of human IgG in the presence and absence of beta cyclodextrin . 1mg/ml, 2.4mg/ml human IgG and 1:1%w/v (1mg/ml, 2.4mg/ml) of human IgG with beta cyclodextrin n=3

Formulation	**(1mg/ml and 2.4mg/ml) Human IgG**		**1:1 %w/v (1mg/ml and 2.4mg/ml) Human IgG beta cyclodextrin**	
Concentration	**1mg/ml**	**2.4mg/ml**	**1mg/ml**	**2.4mg/ml**
T_m(°C) pH 5.5	68.08±2.5	67.47±4.6	68.68±4.4	67.90±2.4
T_{agg} (°C)	78.54	75.00	81.54	75.00
T_m(°C) pH 7.4	71.51± 1.4	70.66+0.35	71.91+1.4	70.66±0.4
T_{agg} (°C)	82.00	76	79.00	76.00

The 1mg/ml concentration of IgG with beta cyclodextrin tend to prolong aggregation temperature from 78.54 degree without beta cyclodextrin, and to 81.54 degree with beta cyclodextrin. Observations on the effect of the excipients on the thermograms with pH 5.5 and 7.4 were unremarkable as increased pH tend to decrease denaturation temperature of IgG at both concentrations. 1:1%w/v IgG beta cyclodextrin at 1mg concentration tend to decrease aggregation temperature to 79°C (Figure 5.8) compared 82°C of IgG in the absence of excipient at pH 7.4

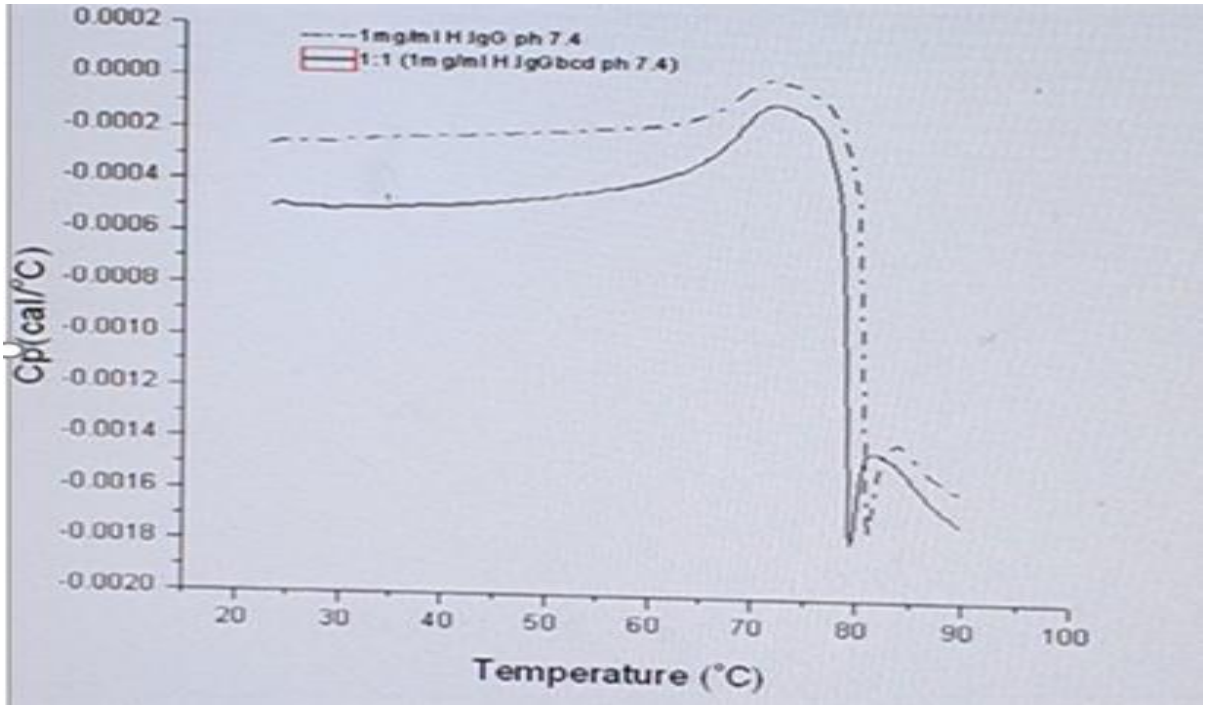


Figure 5.8 Raw data showing reduced aggregation temperature of 1mg/ml human IgG and 1:1%w/v (1mg/ml) of human IgG with beta cyclodextrin at pH 7.4. dark dotted line represents 1mg/ml human IgG; dark line represents 1:1(1mg/ml) IgG with betacyclodextrin.

The presence of beta cyclodextrin in IgG formulations at increased concentration (2.4mg/ml %w/v) showed no effect on the aggregation temperature at a 5.5 pH level (Figure 5.9), as the figure clearly shows formulation for 1mg/ml, 2.4mg/ml, IgG with the addition of betacyclodextrin in a 1:1 concentration. Addition of beta cyclodextrin to 2.4mg/ml at a pH of 5.5 showed little or no changes to the thermogram, as aggregation temperature were both observed at about 76°C temperature.

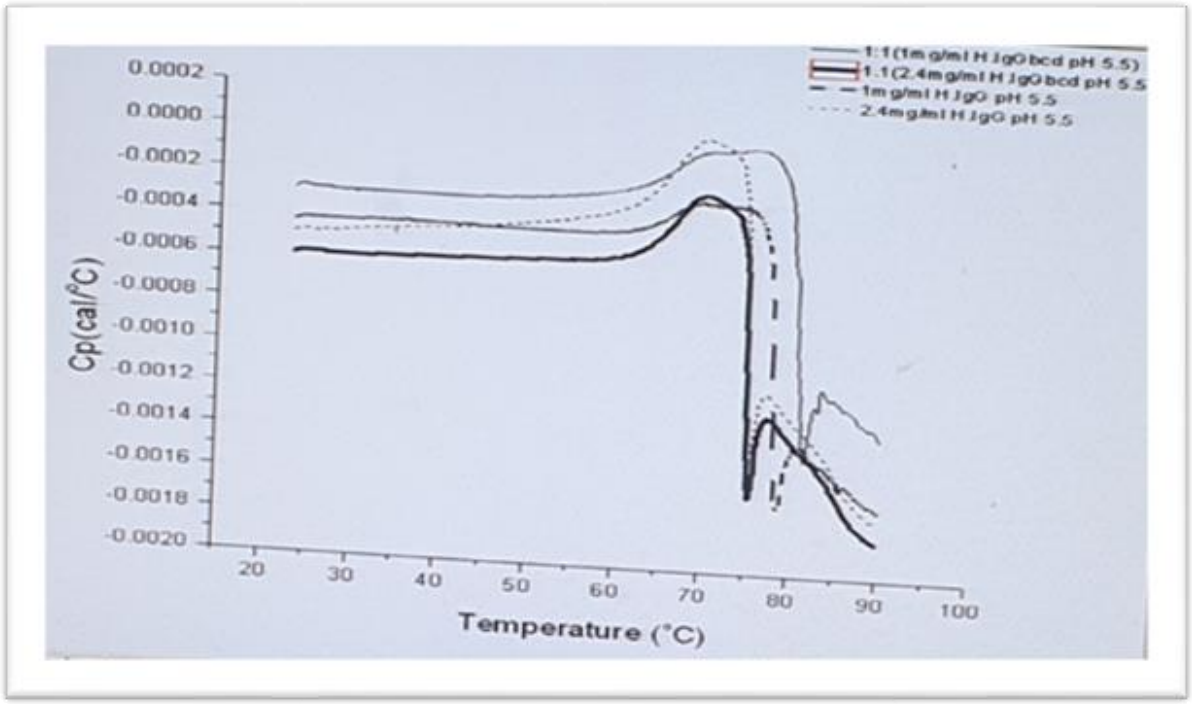


Figure 5.9. 1mg/ml and 2.4mg/ml concentrations of IgG at pH 5.5 in the presence and absence of beta cyclodextrin (thin dark line represent 1:1 (1mg, IgG:Beta cyclodextrin), thin dotted line represents 1:1 (2.4mg, IgG:Beta cyclodextrin), dotted boldened line represents (1mg, IgG), boldened line represents (2.4mg IgG).

The IgG fold has an extremely compact topology and is primarily found in multidomain proteins. It is made up of two beta-sheets that form a barrel. The two beta-sheets are connected by a conserved disulphide bridge in the centre, which appears to increase the structure's stability Feige et al., 2004. Glycosylation, deamidation, oxidation, and disulfide bond reduction are just a few of the post-translational modifications that impact the function of proteins which could potentially result in aggregation and oligomerization (Maverakis et al., 2015). Due to the formation of aggregate and immunogenicity of biotherapeutics, it is essential to keep a close eye on the aggregate profile (Filipe et al., 2010). One of the most frequent types of chromatography is called size-exclusion chromatography (SEC) methods for analysing aggregate content in biotherapeutics.

Proteins are classified in accordance with the hydrodynamic radii of their molecules (Fekete et al., 2014), with larger molecules eluting earlier than smaller ones, with deglycosylated forms, mAb hydrodynamic radii are eluted earlier than those of glycosylated forms. It is now possible to accurately quantify aggregate content in as little as ten minutes using like SEC-HPLC (Diederich et al., 2011). However, there are critical limitations to SEC analysis due to the protein analyte characteristics (Knihtila et al., 2021). SEC-HPLC was used to assess the effect of Beta cyclodextrin on IgG at a low and high pH (5.5;7.4). The pH effect on the solutions were different, at pH 5.5 a strong retention occurred with high resolution and a high peak tailing for the buffer while less resolution and lower tailing was observed at pH 7.4 (Figure 5.10). This confirms observation outlined by Knihtila et al (2021) on the effect of low pH occurring at a strong retention time, a high tailing and resolution also, higher pH occurring at a lesser column retention, lower resolution and lower tailing. It was further stated it could be the effect of repulsion where a protein carries a negative charge at high pH and will be repulsed by the negative charge. Addition of beta cyclodextrin decreased the retention time of IgG and also reduced the monomeric percentage area from 71% to 64%.

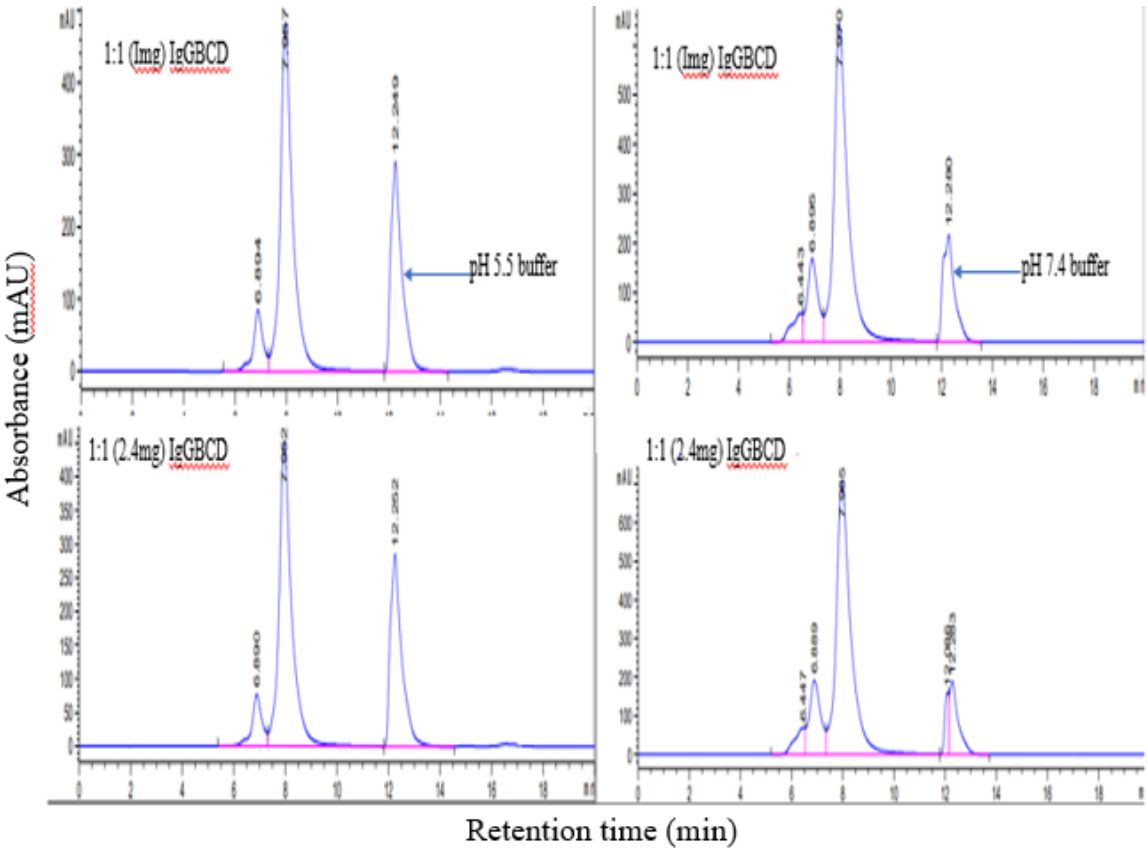


Figure 5.10 Chromatograms showing 1:1(1mg/ml and 2.4mg/ml) concentrations of IgG in the presence of beta cyclodextrin at pH 5.5 and 7.4.

Data obtained from DLS implied that the presence of betacyclodextrin on the heated sample at a lower pH increased the particle size of Human IgG even though aggregation was confirmed. Also, addition of beta cyclodextrin resulted in the formation of larger particles. The large particle size obtained after heating could be as a result of solution conditions (Zhou et al., 2015).

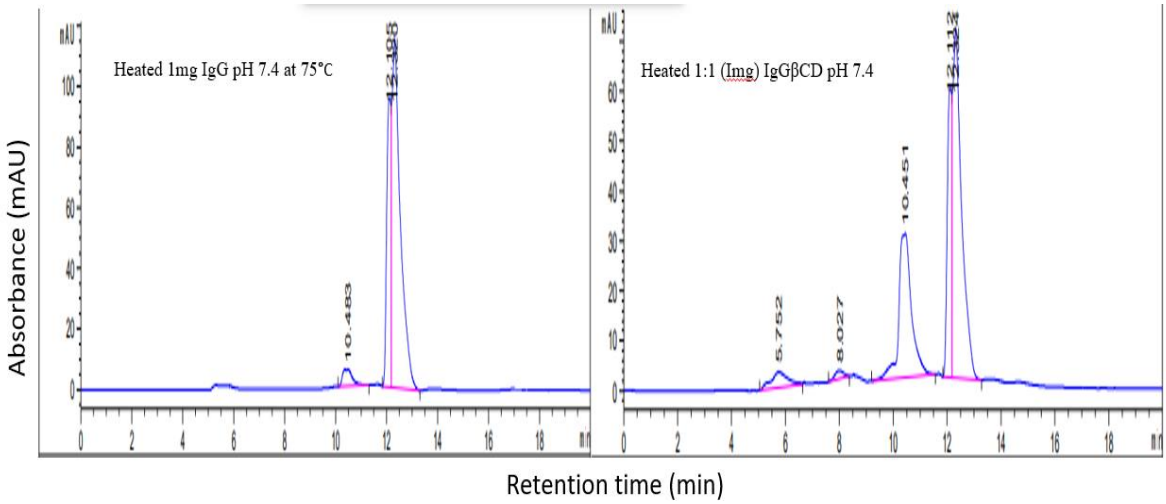


Figure 5.11 Chromatogram confirming aggregation of heated IgG at 75ºC in the presence and absence of beta cyclodextrin at pH 7.4. n=3

The heated IgG (Figure 5.11) showed no peak transition for IgG however addition of beta cyclodextrin to the formulation after heating at 75 degrees exhibited a minor peak at 8.03 min retention time and 41% monomeric percentage area.

Cyclodextrins (CDs) have been reported to be a potential class of anti-aggregation stabilisers, according to the literature. Although the ability of CDs to preserve proteins has frequently been linked to their ability to associate with proteins, evidence has also been presented for their role in preventing proteins from being exposed to water and air. Furthermore, they are an excellent choice for the production of tailored microparticles for the delivery of peptides and proteins through the respiratory system (Milani et al., 2020).

According to the available information in the literature, the ratio of CDs to proteins that is being utilised is extremely variable. To function as an ideal stabiliser, it will be necessary to use higher and lower concentrations of CDs, depending on whether dehydration is the primary stress factor or whether surface denaturation is present. (Castellanos at al., 2006). As a logical approach, such a ratio should be tuned in accordance with the protein type and the manufacturing technique involved. Despite a large number of articles indicating the

suppression of protein aggregation by CDs for antibodies, which are considered to be one of the most important groups of biopharmaceuticals, there are few empirical data to support this claim (Branchu et al., 1999; Milani et al., 2020).

5.4.5. Effect of sources / species on IgG thermal stability

The human and bovine IgG were investigated to determine how the sources or species of these protein react to thermal stability. Investigation on the source of antibody showed some effect on the Tm value and aggregation. When the source of antibody was compared alongside the pH 5.5 used in the formulation, bovine antibody showed higher denaturation temperature compared to human IgG, this could be as a result of the positions in the amino acids, however the exothermic reaction occurred at almost the same temperature confirming aggregation (Figure 5.12).

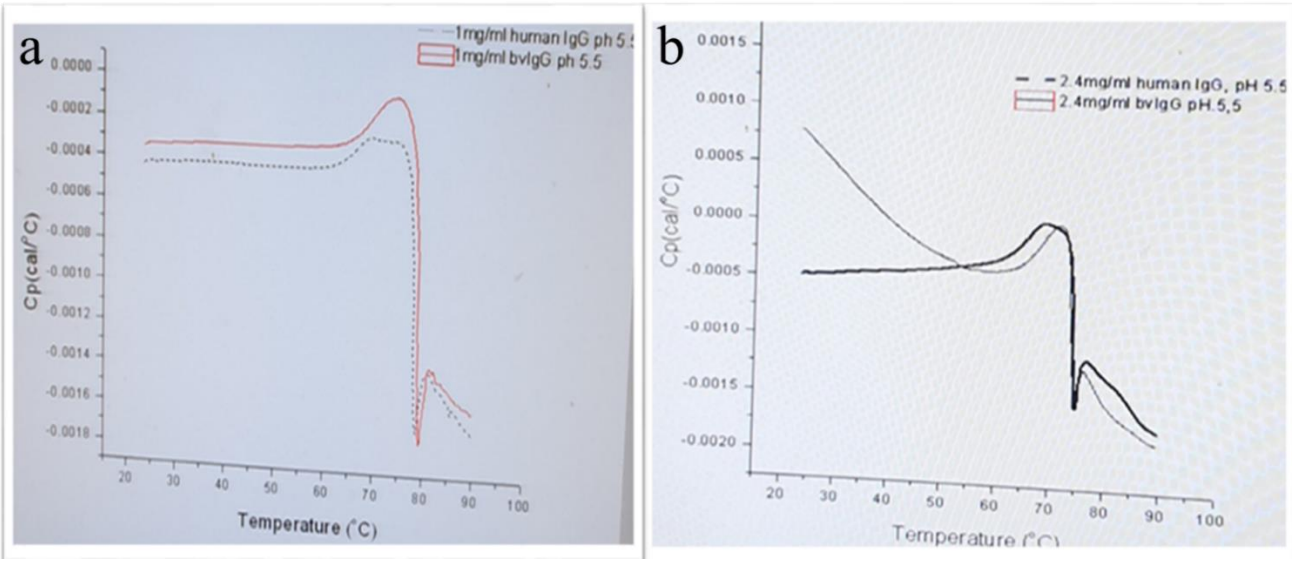


Figure 5.12 Comparison of raw DSC thermogram of human and bovine IgG (1mg/ml,2.4 mg/ml) in the presence and absence beta cyclodextrin in a 10-mM sodium citrate, 140mM NaCl buffer, pH 5.5(a)represents 1mg of human and bovine IgG with dotted black line being bovine IgG and red line representing human IgG and (b) represents 2.4mg of human and bovine IgG with boldened line representing human IgG and thin line representing bovine IgG.

The addition of beta cyclodextrin to bovine IgG (Figure 5.13) at a lower pH showed little or no effect on both the denaturation temperature and aggregation temperature. This is an indication that beta cyclodextrin neither improved stability nor aggregation.

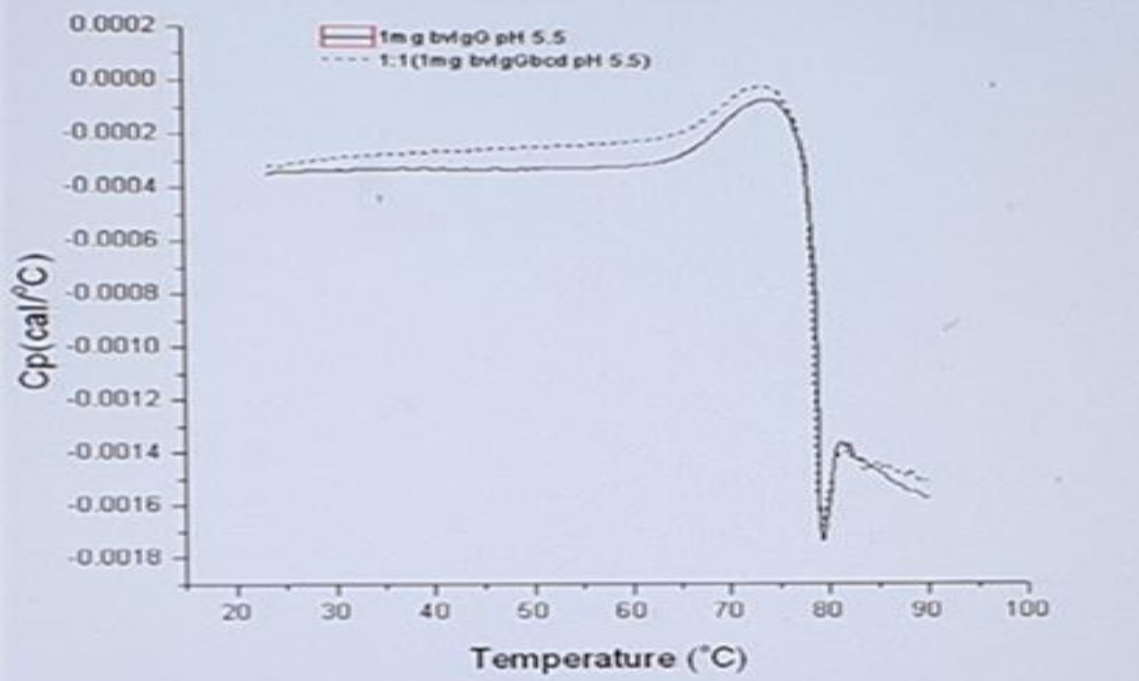


Figure 5.13 Raw data showing little or no effect of denaturation and aggregation temperature of 1mg bovine IgG and1:1%w/v (1mg) bovine IgG beta cyclodextrin at pH 5.5 where dotted dark line represents 1mg bovine IgG, dotted dark line represents bovine IgG with beta cyclodextrin.

There may be a significant difference in the stability of protein depending on the source and manufacturer of the protein (Brange et al., 1992a; Wang, 1999). The purity of the protein is a major factor that influences the stability of the protein; the presence of even a small amount of metal ions, enzymes or other contaminants may have an impact on the stability of the protein. Therefore, one of the most important phases in regulating the quality of proteins is the process of protein purification. The rate at which high-molecular-weight products are formed in proteins such as human insulin preparations in neutral solutions might vary by up to one hundred percent from one manufacturer to the next (Brange et al., 1992a), the extent of protein

degradation and the method by which it occurs vary greatly depending on the purification scheme that is used (Wang 1999). Different methods of purification may be necessary for recombinant protein and plasma-derived protein, which will have an impact on the stability of the proteins. Interactions between proteins and water are crucial to the proper functioning, folding, and structural integrity of proteins in their biological context, the water surrounding the protein is a contributory factor to the major forces necessary to stabilise protein folded conformation. These forces include hydrogen bonds, salt bridge and Van der Waals interaction. Minimal presence of water gives rise to an increased Tm and a decreased ΔH. Hydration effect on stabilization of protein decreases with an increase in the degree of hydration (Zaks 1992). DSC has been utilised to evaluate the likelihood of degradation; nevertheless, its capacity to determine the course of degradation has been restricted due to its limitations. When a monoclonal antibody is produced in buffers that have a small range of pH slightly varying concentrations of salt, the principal thermal transition temperatures, also known as Tm, of the antibody are typically indistinguishable from one another. As a result, in order to determine which formulation options hold the greatest promise within the constraints of the therapeutic product development timeframe, formulations need to be tested under accelerated circumstances such as high shear, elevated temperature, and freeze-thaw cycles (Liu et al., 2006).

Exposing protein to gas-liquid interfaces during formulation and filling may create denaturation. To resist aggregation and adsorption to surfaces, non-ionic surfactants are often used in protein formulations. Surfactants like Tween 80, Pluronic F-68, and Brij 35 have exhibited induced aggregation, but they are not stable against thermal stress, according to DSC reports, a higher concentration will cause the molecule to become unstable. Sugars are known for aggregation prevention of a variety of protein with HP-SEC detecting soluble aggregates in many proteins, including insulin and antibody (Wang et al., 2017). Low amounts of soluble

aggregates in pharmaceutical products will generally be accepted as long as the substance is stable, and the soluble aggregates do not transform into insoluble forms. The immunogenicity of the therapeutic protein can change as soluble aggregates increase. In certain cases, such as insulin and IL-1β, aggregates can be used. FTIR, Raman, and electron spin resonance spectroscopy, as well as light scattering methods, can be used to detect insoluble aggregates (UV absorption). Protein aggregation is the most frequent and concerning symptom of protein instability, occurring at virtually any point of drug production.

Protein aggregation, along with other physical and/or chemical instabilities in proteins, continues to be a significant roadblock in the development of new protein drug candidates. Despite the fact that a number of approaches have been designed to inhibit protein aggregation, the end results for certain proteins are always unsatisfactory. Part of our limited success can be attributed to a lack of knowledge of the protein aggregation mechanism. An important task during formulation is finding the right excipients to mitigate (reduce) protein aggregation and self-association. The success story of monoclonal antibodies have been due to their high binding affinity and specificity and the availability of humanised forms that attenuate (mitigate or reduce) immunogenic processes and responses, but aggregated antibodies are sometimes observed in the formulation processes at mostly high concentrations greater than 100mg/ml. Other factors include changes in the pH of the solution, shaking, freeze thawing and temperature fluctuations (Kheddo et al., 2014).

Immunogenicity has caused aggregation levels in monoclonal antibody drug to be crucial (Diederich et al., 2011). Aggregates of monoclonal antibodies are frequently the most abundant product-related impurity. The purification process must ensure that aggregate levels in the final drug product are kept to an acceptable level. The SEC approach accurately

describes the types and levels of aggregates in the protein sample, during product formulation development, changes in the extent and types of protein aggregation are a major concern. SEC is a useful tool for stability monitoring because of its low run times and quantitative reproducibility. Large numbers of samples can be generated by stability protocols with numerous formulations, manufacturing batches, storage conditions, and time-points, and the 15–20min run-times achievable by HPLC columns and systems can give better sample throughput (Hong et al., 2012).

5.5 Conclusion

Adding the right excipients, such as sugar to antibody solutions is typically necessary to solve stability issues when subjected to mechanical or thermal stress. In theory, cyclodextrins can stabilise proteins since they have modest surface activity (similar to surfactants) and a sugar-based structure.

Therefore this chapter investigated the influence of beta cyclodextrin on low concentrations of IgG formulations.

The thermogram of monoclonal antibody (IgG) showed a transition followed by a sharp aggregation peak. It was observed that the thermograms showed different reactions to pH. An increase in pH (7.4) was not seen to be different when compared to pH 5.5. Similarly, observations on particle size analyses did not show obvious contrast in the different pH applied considering the standard deviation values in table 5.2 and 5.3 on page 176. Results from the Dynamic light scattering confirm the samples were aggregated from the DSC after heating. An Increase in concentration affected the denaturation temperature negatively.

The presence of Beta cyclodextrin at lower concentration appeared to have a positive effect on IgG denaturation temperature and aggregation temperature, however, increase in pH reduced the aggregation temperature.

When comparing the source of IgG on thermal stability, bovine IgG showed very little difference in stability compared to human IgG as the denaturation temperature was slightly higher at pH 5.5. However, addition of betacyclodextrin shows little or no effect to the denaturation temperature.

Data obtained from HPLC chromatograms confirms the presence of aggregation after sample heating from 70°C.

CHAPTER SIX

6.0 GENERAL CONCLUSION AND FUTURE WORK

6.1 General Conclusion

The goal of protein therapeutic formulation research is to create effective products in which the protein remains in solid and solution form, maintaining its stability and activity throughout its shelf life (Raut and Kalonia, 2016). Proteins and polypeptide molecules have established potential biological activity when they are tested against a number of diseases; these drugs have to remain stable and should be able to produce therapeutic effect. The native state of therapeutic proteins has a limited physical stability. The parenteral route is a typical way of administration because proteins tend to be poorly absorbed by the mouth, not withstanding the fact is that frequent injections are inconvenient and can result in decreased compliance. There are other delivery methods that are appealing, and indeed the lungs, with their enormous absorptive surface, are one of the methods that provide this. Small particle sizes required for pulmonary distribution, as well as the increased moisture content in the respiratory tract, create challenges for the administration and formulation of protein therapeutic.

Spray drying is a good way to make particles that are ideal or suitable enough for inhalation (Hulse et al., 2009). It is a simple and convenient way to make protein solutions by turning them to powder. The approach improves nanoparticle physicochemical stability. Spray drying is a one-step procedure utilized in making a wide range of powders with specific qualities for specific uses (Rajesh et al., 2018).

Spray drying and electrospray techniques were employed in this research for thermal stability of lysozyme and insulin investigation. However, ESD only was used for lysozyme investigation. The formulations were prepared in form of solution and further dried using the Buchi 290 spray dryer and subsequently characterized with differential scanning calorimetry

(DSC), dynamic light scattering (DLS), scanning electron microscope (SEM). Spray dry technique in the absence of beta cyclodextrin and pluronic F-127 impacted the denaturation temperature of lysozyme and insulin in a negative way. It is possible to think of the stability of the protein in a variety of ways. Structural and thermal stability are referred to as protein's structural ability to resist denaturation by heat.

Differential scanning calorimeter was applied to investigate protein stability as a function of solvent pH, temperature and concentrations of excipients. The temperature is dependent on the conditions of the solutions and the protein's molecular structure(Garidel et al., 2020). Parts of the excipients used include amino acids, sugars, and salts.

Thermal transition temperature (Tm) measurements of protein solutions in the absence and presence of additives carried out using these methods. Increases in Tm levels are a sign that thermal stability has been improved. Because of the prolonged shelf life and reduced danger of microbial contamination that thermostable enzymes provide, thermal protein stabilization is critical in companies that use these enzymes.

The capacity of a protein to withstand the formation of aggregates in solution through the process of self-association; also known as colloidal stability, is defined as solution stability (James et al., 2012). It has been used for decades in stability testing of protein solutions by means of light scattering, excipients present help to influence the behaviour of a protein in solution (Jorgensen et al., 2009). Multiple strategies and techniques have been applied to characterize thermal stability, conformation and protein aggregation of these proteins. The obtained results are combined from various perspectives and instrument to give a general assessment of the proteins physiochemical properties (Hawe et al., 2012). An essential indicator of changes in the chemical and physical characteristics is a peak. The peak could either be shifting or lost, it further indicates changes in the drug nature. Since protein

aggregation can often result in a decrease in biological activity, an increase in immunogenicity, or toxicity, excipients are commonly used to prevent this (Wang, 1999). Excipients play an important function in keeping formulation of protein physically stable (Ohtake et al., 2011). Certain excipients can have a dual effect depending on their concentration and nature; for example, preferentially excluded co-solutes increase protein conformational stability in solution, but when the rise in the chemical potential of the protein in the solution phase is greater than that of the protein in the solid phase, phase separation (also known as reduced solubility) can be encouraged (Timesheff, 2002). Excipients stabilise proteins via modifying conformational stability or modulating protein–protein interactions in solution. Excipients, including things like amino acids polyols, sugars, polymers, salts, surfactants and their derivatives are employed in therapeutic formulations of protein to improve physical stability (Karmezell et al., 2011). In this regard, beta cyclodextrin and Pluronic F-127 were the experimental excipients used in this study, to investigate the influence on Lysozyme, Insulin and Monoclonal antibody. Results obtained from the experimental techniques applied on Lysozyme formulations resolved that beta cyclodextrin and Pluronic F-127 exhibited a form stability in various significant ways. From the biological activity data with both drying techniques applied, there were promising observation with the excipients. The presence of beta cyclodextrin in spray dry lysozyme maintained biological activity. Adding beta cyclodextrin and pluronic F-127 maintained the denaturation temperature of spray dry lysozyme.

Furthermore, it could be stated that a combination of beta cyclodextrin and pluronic could increase the activity and stability of lysozyme using the right technique and formulation as the electrosprayed technique displayed. Spraydried lysozyme with excipients showed some specific actions in the biological activity, however, it showed lack of stability using the DSCs apparent temperature. Studies show that proteins can have some activity to a certain level, while part of the structure might have been affected by degradation or dehydration. Dehydration during the process of drying could possibly be the reason for the

protein's altered structure, degradation of lysozyme was not ruled out in this study as heat could have been a major physical stress that affected the formulations. For insulin characterization, beta cyclodextrin and pluronic F-127 showed possible interactions as well. Beta cyclodextrin was a better excipient in percentage yield comparison as the spraying conditions used appeared favourable. The effect of excipients on stability of other proteins have been addressed. Shenoy et al. (2000) investigated the tehalose, lactose and sucrose on stability of glucose oxidase and lipase, in amorphous and crystallised form. It was resolved that the use of lower excipients amount better stabilised some form of protein though in crystallised form when stored (Haj-Ahmed et al., 2013) however, there may be consequences with the use of some excipients in the formulations because of the detrimental implications of the protein's stability. Hulse et al. (2009) showed that trypsin and lysozyme have an effect on the polymorphism behaviour of mannitol when it is being spray dried, which in turn has an effect on the stability of the compound. Polyols and non-ionic surfactants are used commonly as excipients for preventing aggregation (Bishop and Wolford, 2008). Polyols are similar to sugars in that they are used in high concentrations to stabilise proteins during thermal stress and quiescent storage. This is accomplished through the preferential exclusion of polyols from the surface of the protein, leading to protein hydration increase with a decreased tendency for the protein to unfold. (Jorgensen at al., 2009). Despite this, proteins remain unstable when subjected to mechanical agitation and interfacial damage. A small number of surfactants like polysorbates stabilise proteins by competitive adsorption, preventing the protein from reaching the interface and being damaged (Manning et al., 2010). Consequently, various stresses such as stirring, freeze-thawing, nebulization or shaking are effectively prevented by them. However, long-term storage may have detrimental effects, as protein oxidation may be accelerated (Hartl et al., 2013).

High sensitivity differential scanning calorimetry (HSDSC) is an effective method for determining the viability and thermal stability of protein (Lipiainne et al., 2018; Thiagarajan

et al., 2016). It was used to determine the thermal analysis of the proteins. In this research it was used on Lysozyme, Insulin and Monoclonal antibodies. DSC data is frequently utilised to identify and evaluate formulations with the best stability structure. The overall temperature denaturation transition of the proteins was made up of different types and shapes of peaks. Lysozyme had a single peak, some insulin formulation had double peaks and some monoclonal antibody formulations, triple peaks. For most monoclonal antibodies in literatures, the heat capacity function has three peaks, which correspond to the CH2, CH3, and Fab domains as described by Garber and Demarest (2007) and according to numerous DSC investigation however, in this study instead of the CH3 domain, exhibiting a third transition peak, there was an appearance of aggregated peak. Different IgG subclasses, such as IgG1, 2, 3, and IgG4, exhibit this behaviour (Garber and Demarest, 2007). The first transition, which is the least stable domain, belongs to the CH2 domain; another the greatest peak corresponds to the Fab domain; and the third peak, which appear almost identical in size to the CH2 domain, refers to the CH3 domain. Denaturation of CH2-CH3 pair may sometimes occur before the Fab, or even at greater temperatures.

Thermal analysis of Lysozyme samples showed that electrospray technique improved the apparent denaturation temperature making the electrosprayed sample more stable. The presence of excipients also suggests an increase in thermal stability as observed from the denaturation temperature report. The presence of excipients in lysozyme formulations in the absence of drying negatively affected the denaturation temperature making lysozyme unstable. Insulin formulation showed Pluronic F-127 impacted the thermal stability positively compared the Beta cyclodextrin using phosphate buffer saline, on the other hand, insulin in HCl prolonged the aggregation temperature. Furthermore, the presence of Beta cyclodextrin at lower concentration appeared to have resulted in a positive effect on monoclonal antibody

aggregation and denaturation temperature however, increase in pH reduced the aggregation temperature.

Drying technique improved the thermal denaturation of lysozyme also, both technique and excipient could enable drying operations with satisfactory powder yields being achieved in their unique forms. Beta cyclodextrin appeared to be a better excipient for maintaining stability and activity of lysozyme formulation in this study. It also presented an advantage of more efficient drying with spray dry techniques compared to electrospray.

It is possible to declare that the combination of beta cyclodextrin and pluronic F-127, when applied with the appropriate methodology and formulation, has the potential to boost both the activity and stability of protein as the electrosprayed technique has shown. The most difficult step in developing a protein formulation is stabilising a protein to reach an appropriate shelf life. Excipients are widely used to stabilise proteins due to the complexities of structural changes. Sugars, polyols, surfactants, salts, polymers and amino acids are all common protein stabilisers. Unfortunately, due to lack of a clear and conclusive understanding of protein-cosolute interactions and proteins' numerous inactivation processes, there is no one approach to follow in selecting an appropriate stabilizer. Traditional protein stabilisers may only boost protein stability to a limited extent depending on the protein. As a result, new forms of protein stabilisers must be investigated further. One apparent route is to look for additional cellular components in hyperthermophilic animals or cellular accumulants under varied stressful situations that could help stabilise proteins. In conclusion, the most challenging difficulty in developing a liquid protein pharmaceutical is maintaining the protein's biological activity over an adequate shelf life. Unfortunately, there is no standard approach that works for all in creating such a product.

The sugar and surfactant used (Beta cyclodextrin and Pluronic F-127) at 1:1 and 1:5 concentration demonstrated several promising qualities on lysozyme with the drying technique

applied. Electrospray technique in the presence and absence of excipients improved lysozyme stability. In general, Beta cyclodextrin used with every model of protein (insulin, Immunoglobulin) showed a form of possible stability at different concentrations. Electrospray technique was not applied to insulin and IgG formulations as a result of the inability to obtain a clear solution after reconstitution for high sensitivity differential scanning calorimetry.

The concrete conclusion of this research shows that drying techniques (both spray drying and electrospray) positively improved the stability of lysozyme with the addition of beta cyclodextrin in different unique ways.

Beta cyclodextrin appeared to be a better excipient for spraying insulin when comparing the percentage yield obtained from this research. The conditions used for spray drying appeared unfavourable for insulin formulation.

Investigation on thermal stability of insulin showed that pluronic F-127 appeared to have impacted on the stability of insulin using Phosphate buffered solution while beta cyclodextrin had no effect on the stability of insulin at pH 1.2 HCl. On the other hand, insulin in the presence of beta cyclodextrin in HCl prolonged the aggregation temperature compared to pluronic F-127.

Furthermore, 60ºC/hr scan rate is a prefered temperature scan rate as it delayed aggregation compare to 10ºC/hr.

Investigation of thermal stability of IgG observed that the presence of Beta cyclodextrin at lower concentration appeared to have a positive effect on IgG denaturation temperature and aggregation temperature, however, increase in pH did not show a difference in the aggregation temperature.

When comparing the source of IgG on thermal stability, bovine IgG appeared to be more stable than human IgG as the denaturation temperature was higher at pH 5.5 compared to human

IgG. However, the addition of betacyclodextrin showed little or no effect to the denaturation temperature.

Physical examination for lysozyme with and without excipient showed no evidence of discoloration compared to insulin dissolved in HCl, in which discoloration was evident. However, a cloudy formulation was observed for monoclonal antibody without beta cyclodextrin. It was further noted that the addition of beta cyclodextrin to IgG formulation reduced the cloudy formation in the liquid formulations.

6.2 Future Work

Beta cyclodextrin (βCD) and Pluronic F-127 demonstrated many roles in protein stability over a concentration of Protein:βCD, ratios 1:1 and Protein:Pluronic F-127 ratio1:1; 1: 5. It was observed that both excipients could stabilise lysozyme, Insulin and IgG molecules at the same concentrations in different and unique ways. The morphology and particle size values were shown to be influenced by the constituents of the formulation and the amounts, including the molecular-dependent stabilisation that the excipients provide. it is difficult to pinpoint one major method of action as the major effect of the excipients on protein stabilisation. The most relevant range was 1:1 ratio of lysozyme to beta cyclodextrin, 1:1 insulin to beta cyclodextrin and 1:1 IgG to beta cyclodextrin. From a formulation standpoint, it would be ideal to introduce different concentrations ratios at a (low, mid and high) level rather, certain softwares such as minitabs that suggest the ratios may be considered.

Also, a higher concentration of protein with a lower excipient concentration will be ideal from a 1:1 to a 5:1 concentration. In addition, more research by factorial design may be used to mechanically characterize possible interactions between the excipients and other substances.

The basic characteristics of protein must be thoroughly investigated in the development of liquid protein compositions, because the data gathered can be used to solve formulation issues.

In addition, screening procedures should be utilised with prudence to assist rapid formulation development. Screening approach such as the use of Circular dichroism technique and Fourier Transform Infra-red Spectroscopy for structural characterization may be considered. Further detailed SEC-HPLC characterization could be experimented.

A combination of spray drying technique with excipient on Lysozyme positively improved stability, such technique may be applied on monoclonal antibody.

CHAPTER SEVEN

7.0 REFERENCES

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CHAPTER EIGHT

8.0 Appendix

Appendix i: All formulations compositions

1. 1%w/v unprocessed lysozyme
2. 1%w/v spraydried lysozyme
3. 1%w/v electrosprayed lysozyme
4. 1%w/v spraydried lysozme with 1%w/v betacyclodextrin
5. 1%w/v spraydried lysozme with 1%w/v betacyclodextrin and 5% pluronic
6. 1%w/v spraydried lysozme with 5% pluronic
7. 1%w/v unprocessed insulin
8. 1%w/v spraydried insulin
9. 1%w/v spraydried insulin with 1%w/v betacyclodextrin
10. 1%w/v spraydried insulin with 1%w/v betacyclodextrin and 1%w/v pluronic
11. 1%w/v insulin with 1%w/v pluronic
12. 1%w/v native insulin
13. 1%w/v insulin with 1%w/v betacyclodextrin
14. 1%w/v insulin with 1%w/v betacyclodextrin and 1%w/v pluronic
15. 1%w/v insulin with 1%w/v pluronic
16. 1%w/v unprocessed monoclonal antibody
17. 1%w/v monoclonal antibody with 1%w/v betacyclodextrin
18. 1%w/v monoclonal antibody with 1%w/v betacyclodextrin and 1%w/v pluronic
19. 1%w/v monoclonal antibody with 1%w/v pluronic

Appendix ii: Preparation for spray dry lysozyme solution

Dissolve 1g of Lysozyme in 100 ml of water

Dissolve 1g of Lysozyme + 1g of Betacyclodextrin in 100ml of water

Dissolve 1g of Lysozyme + 5g of Pluronic in 100ml of water

Dissolve 1g of Lysozyme + 1g Betacyclodextrin + 5g Pluronic in 100ml of water

Appendix iii: Preparation for electrospray lysozyme solution

Dissolve 1g of Lysozyme in 60 ml Ethanol + 40ml of water

Dissolve 1g of Lysozyme + 1g of Betacyclodextrin in 60ml Ethanol + 40ml of water

Dissolve 1g of Lysozyme + 5g of Pluronic in 60ml Ethanol + 40ml of water

Dissolve 1g of Lysozyme + 1g Betacyclodextrin + 5g Pluronic in 60 ml Ethanol + 40ml of water

No good spray observed. Formulation changed to the below

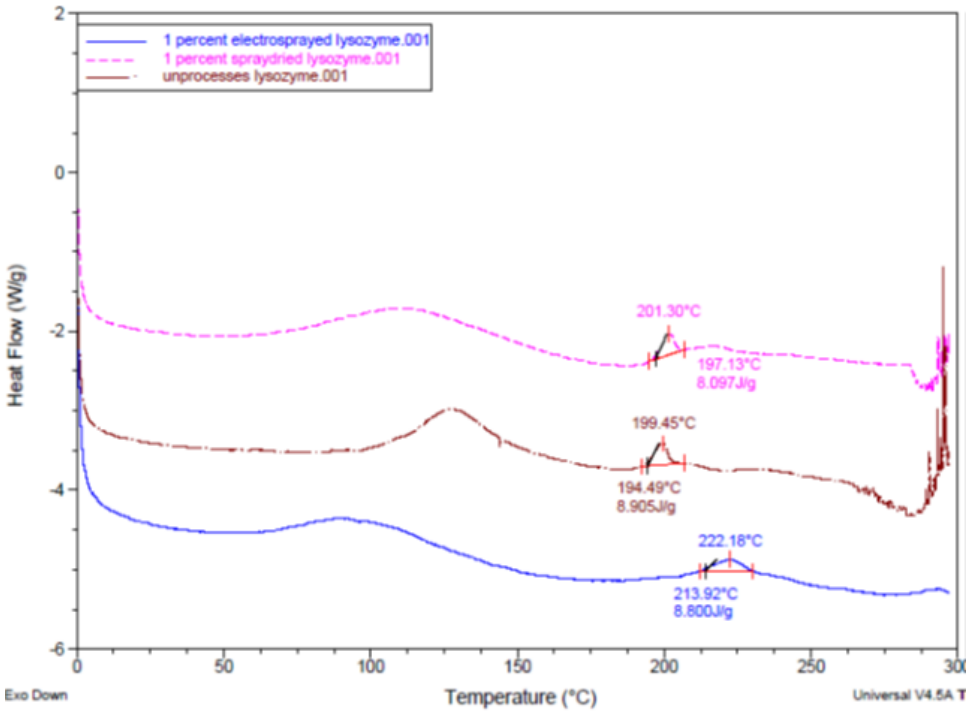
Dissolve 1g of Lysozyme in 80 ml Ethanol + 20ml of water

Dissolve 1g of Lysozyme + 1g of Betacyclodextrin in 80ml Ethanol + 20ml of water

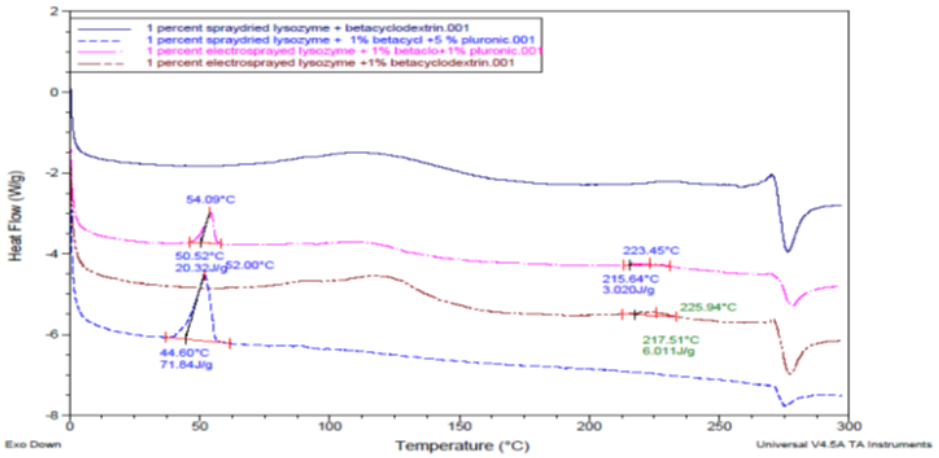
Dissolve 1g of Lysozyme + 1g of Pluronic in 80ml Ethanol + 20ml of water

Dissolve 1g of Lysozyme + 1g Betacyclodextrin + 1g Pluronic in 80 ml Ethanol + 20ml of water

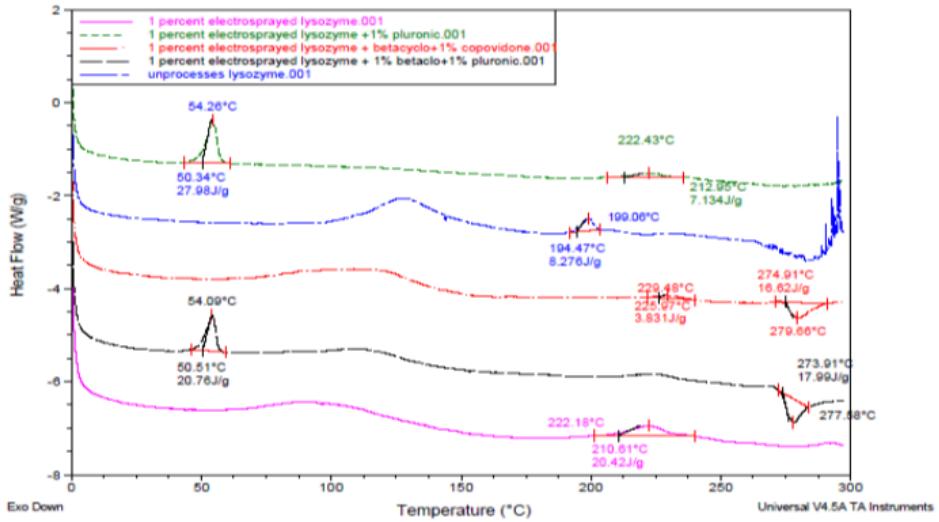
Appendix iv: DSC thermograms for lysozyme samples with spray dry and electrospray techniques



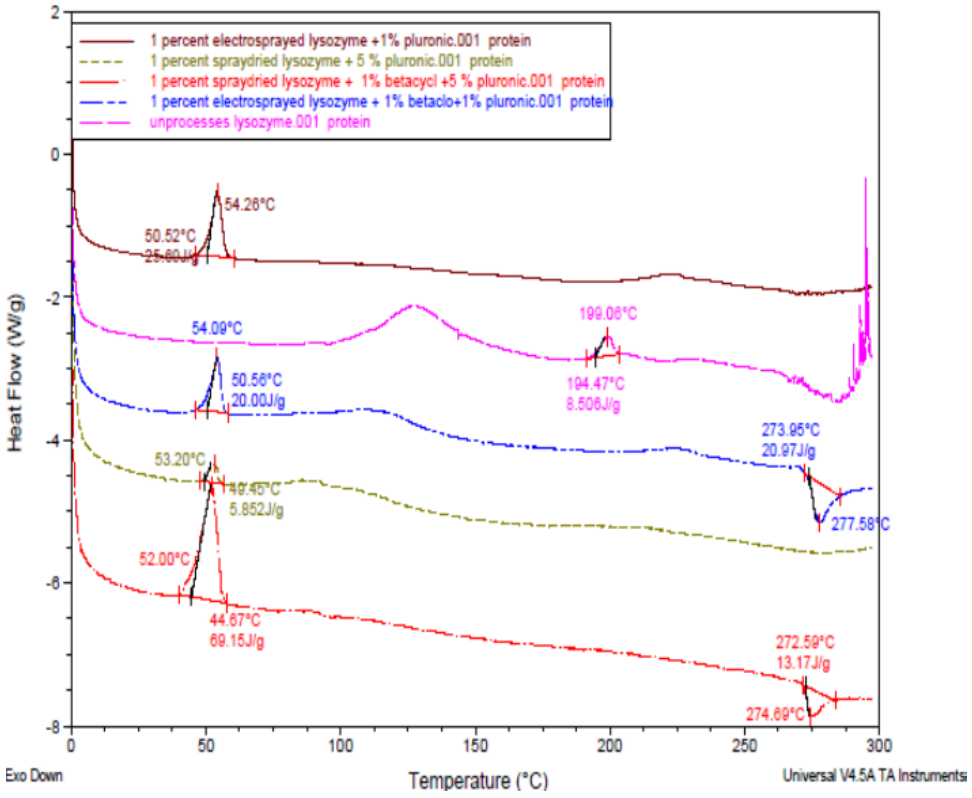
Thermogram showing all unprocessed, spraydried and elecrosprayed lysozyme.

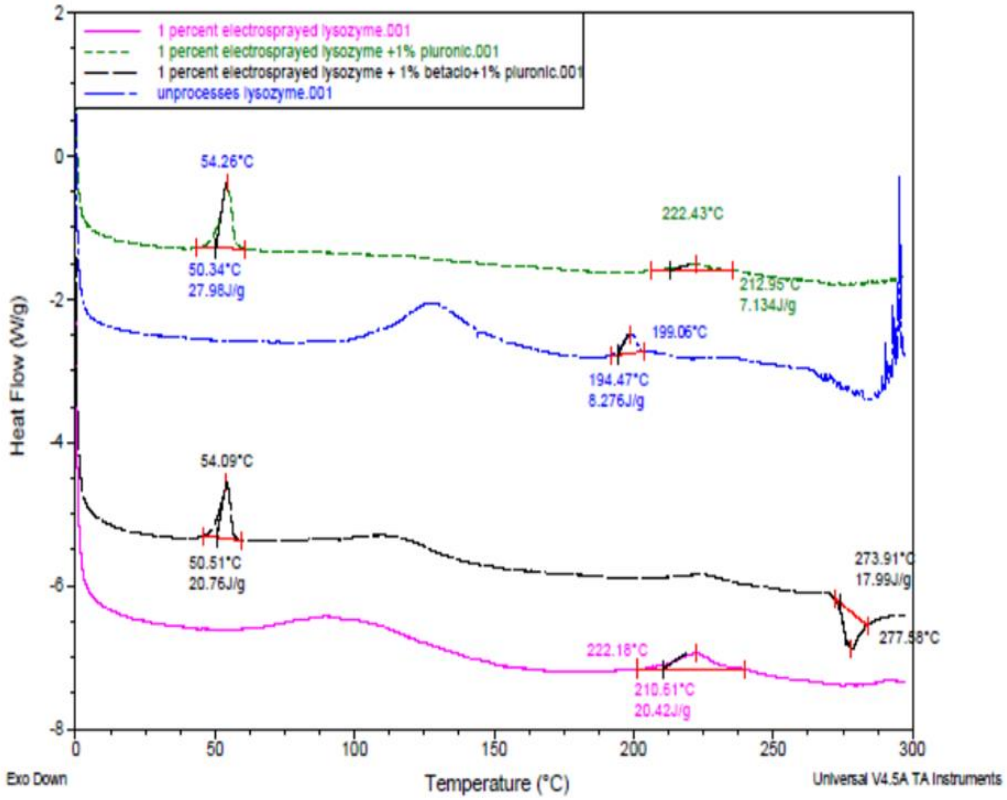


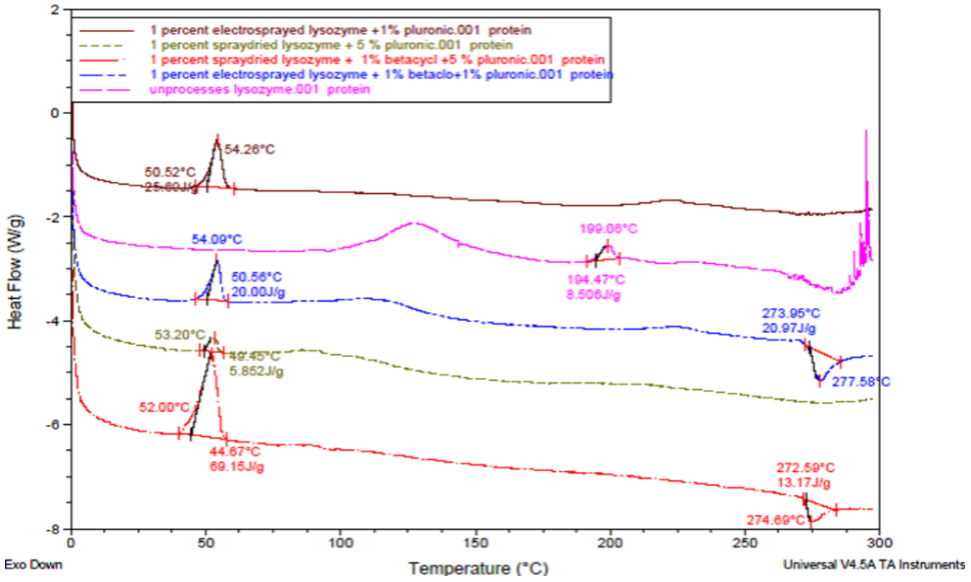
Thermogram showing spray-dried and electrosprayed lysozyme with excipients.



Showing the comparison between unprocessed and electrosprayed lysozyme with and without excipient


1 percent electrosprayed lysozyme +1% pluronic.001 protein
1 percent spraydried lysozyme + 5 % pluronic.001 protein
1 percent spraydried lysozyme + 1% betacycl +5 % pluronic.001 protein
1 percent electrosprayed lysozyme + 1% betaclo+1% pluronic.001 protein
unprocesses lysozyme.001 protein
54.26°C
50.52°C
25.80J/g
54.09°C
199.06°C
194.47°C
8.506J/g
50.56°C
20.00J/g
273.95°C
20.97J/g
53.20°C
48.45°C
5.852J/g
277.58°C
52.00°C
44.67°C
69.15J/g
272.59°C
13.17J/g
274.69°C
Heat Flow (W/g)
Temperature (°C)
Exo Down
Universal V4.5A TA Instruments


1 percent electrosprayed lysozyme.001
1 percent electrosprayed lysozyme +1% pluronic.001
1 percent electrosprayed lysozyme + 1% betaclo+1% pluronic.001
unprocesses lysozyme.001
54.26°C
50.34°C
27.98J/g
222.43°C
212.95°C
7.134J/g
199.06°C
194.47°C
8.276J/g
54.09°C
50.51°C
20.76J/g
273.91°C
17.99J/g
277.58°C
222.18°C
210.61°C
20.42J/g
Heat Flow (W/g)
Temperature (°C)
Exo Down
Universal V4.5A TA Instruments



Figures showing thermogram of unprocessed, spray-dried and electrospray lysozyme with excipients

Appendix v: Biological Activity for Lysozyme

Table Showing the Biological Activity of Formulations.

BIOLOGICAL ACTIVITY OF FRESH AND SPRAY-DRIED LYSOZYME 2							
TIME	**0 MIN**	**1 MIN**		**2 MINS**		**3 MINS**	
BLANK	0.990	0.988	0.002	0.987	0.001	0.984	0.003
FOR FRESH LYSOZYME SAMPLE							
0.01g/25ml was used for the test	0.786	0.570	0.216	0.441	0.129	0.383	0.058
	0.768	0.566	0.202	0.450	0.116	0.384	0.066
	0.774	0.559	0.215	0.437	0.122	0.379	0.058
	0.764	0.561	0.203	0.449	0.112	0.389	0.060
	0.733	0.530	0.203	0.439	0.091	0.388	0.051
TOTAL			1.039		0.570		0.293
AVERAGE			0.208		0.114		0.059

ACTIVITY			1029.000		565.000		278.000
SPRAY-DRIED SAMPLES							
LYSOZYME (1% LYSOZYME)							
0.01g/25ml buffer was used for the test	0.913	0.721	0.192	0.567	0.154	0.474	0.093
	0.873	0.587	0.286	0.454	0.133	0.365	0.089
	0.856	0.575	0.281	0.493	0.082	0.413	0.080
	0.753	0.528	0.225	0.423	0.105	0.360	0.063
	0.843	0.623	0.220	0.495	0.128	0.420	0.075
TOTAL			1.204		0.602		0.400
AVERAGE			0.241		0.120		0.080
ACTIVITY			1194.000		597.000		385.000
% activity of lysozyme			116.035		105.664		138.489
FOR 1% LYSOZYME + 1% B. CYCLODEXTRIN							
0.02g/25ml buffer was used for the test	0.744	0.549	0.195	0.440	0.109	0.384	0.056
	0.667	0.509	0.158	0.419	0.090	0.365	0.054
	0.654	0.508	0.146	0.420	0.088	0.368	0.052
	0.673	0.504	0.169	0.416	0.088	0.364	0.052
	0.714	0.550	0.164	0.451	0.099	0.398	0.053
TOTAL			0.832		0.474		0.267
AVERAGE			0.166		0.095		0.053
ACTIVITY			822.000		469.000		252.000
% activity of lysozyme			79.883		83.009		90.647
1% LYSOZYME + 5% PLURONIC							

0.07g/25ml buffer was used for the test	0.637	0.411	0.226	0.339	0.072	0.295	0.044
	0.626	0.410	0.216	0.339	0.071	0.298	0.041
	0.554	0.382	0.172	0.333	0.049	0.292	0.041
	0.655	0.450	0.205	0.382	0.068	0.339	0.043
	0.540	0.382	0.158	0.320	0.062	0.276	0.044
TOTAL			0.977		0.322		0.213
AVERAGE			0.195		0.064		0.043
ACTIVITY			967.000		317.000		198.000
% activity of lysozyme			93.975		56.106		71.223
1% LYSOZYME + 1% B. CYCLODEXTR + 5% PLURONIC							
0.06g/ 25ml buffer was used for the test	0.516	0.418	0.098	0.378	0.040	0.344	0.034
	0.724	0.583	0.141	0.498	0.085	0.445	0.053
	0.557	0.454	0.103	0.401	0.053	0.364	0.037
	0.603	0.458	0.145	0.400	0.058	0.361	0.039
	0.766	0.673	0.093	0.600	0.073	0.542	0.058
	0.646	0.500	0.146	0.451	0.049	0.406	0.045
	0.633	0.497	0.136	0.421	0.076	0.378	0.043
	0.723	0.603	0.120	0.528	0.075	0.473	0.055
TOTAL			0.982		0.509		0.364
AVERAGE			0.123		0.064		0.046
ACTIVITY			603.75		313.125		212.5
% activity of lysozyme			58.67346939		55.42035398		76.43884892

ELECTRO-SPRAYED LYSOZYME							
TIME	**0 MIN**	**1 MIN**		**2 MINS**		**3 MINS**	
BLANK	0.561	0.581	(0.020)	0.574	0.007	0.577	(0.003)

Fresh lysozyme	0.786	0.570	0.216	0.441	0.129	0.383	0.058
	0.768	0.566	0.202	0.450	0.116	0.384	0.066
	0.774	0.559	0.215	0.437	0.122	0.379	0.058
	0.764	0.561	0.203	0.449	0.112	0.389	0.060
	0.733	0.530	0.203	0.439	0.091	0.388	0.051
			0.208		0.114	0.385	0.059
			100.208		-34.886		15.059
1% ELECTRO-SPRAYED LYSOZYME							
	0.592	0.273	0.319	0.216	0.057	0.196	0.020
	0.583	0.257	0.326	0.194	0.063	0.165	0.029
	0.584	0.278	0.306	0.194	0.084	0.165	0.029
TOTAL	1.759	0.808	0.951	0.604	0.204	0.526	0.078
AVERAGE	0.4398	0.2020	0.2378	0.1510	0.0510	0.1315	0.0195
ACTIVITY UNIT/MG	-606	-1895	1289	-2115	220	-2228	113
1% ELECTRO-SPRAYED LYSOZYME + 1% BETA CYCLODEXTRIN							
	0.511	0.226	0.285	0.174	0.052	0.152	0.022
	0.560	0.238	0.322	0.175	0.063	0.151	0.024
	0.555	0.259	0.296	0.190	0.069	0.165	0.025
TOTAL	1.626	0.723	0.903	0.539	0.184	0.468	0.071
AVERAGE	0.4065	0.1808	0.2258	0.1348	0.0460	0.1170	0.0178
ACTIVITY UNIT/MG	-773	-2001	1229	-2196	195	-2300	104
FOR 1% ELECTRO-SPRAYED LYSOZYME + 1% PLURONIC							
	0.473	0.276	0.197	0.206	0.070	0.182	0.024
	0.449	0.246	0.203	0.206	0.040	0.164	0.042
	0.489	0.234	0.255	0.174	0.060	0.155	0.019

TOTAL	1.411	0.756	0.655	0.586	0.170	0.501	0.085
AVERAGE	0.3528	0.1890	0.1638	0.1465	0.0425	0.1253	0.0213
ACTIVITY UNIT/MG	-1041	-1960	919	-2138	178	-2259	121
FOR 1% ELECTRO-SPRAYED LYSOZYME + 1% COPOVIDONE	BIOLOGICAL ACTIVITY CANNOT BE PERFORMED. REASON: AGGREGATE WAS OBSERVED IN THE SOLUTION.						
FOR 1% ELECTRO-SPRAYED LYSOZYME + 1% BCYO + 1% PLURONIC							
	0.512	0.251	0.261	0.208	0.043	0.192	0.016
	0.474	0.232	0.242	0.192	0.040	0.176	0.016
	0.490	0.219	0.271	0.183	0.036	0.168	0.015
TOTAL	1.476	0.702	0.774	0.583	0.119	0.536	0.047
AVERAGE	0.3690	0.1755	0.1935	0.1458	0.0298	0.1340	0.0118
ACTIVITY UNIT/MG	-960	-2028	1068	-2141	114	-2215	74
1% ELECTRO-SPRAYED LYSOZYME + 1% BCYO + 1% COPOVIDONE	BIOLOGICAL ACTIVITY CANNOT BE PERFORMED. REASON: AGGREGATE WAS OBSERVED IN THE SOLUTION.						

BIOLOGICAL ACTIVITY OF FRESH AND SPRAY-DRIED AND ELECTROSPRAYED LYSOZYME									
	ABSORBANCE							**IU**	
TIME	**0 MIN**	**1 MIN**		**2 MINS**		**3 MINS**		**60**	**120**	**180**
BLANK	0.953	0.954	-0.001	0.954	0.000	0.954	0.000	0.003	0.000	0.000
FOR FRESH										

LYSOZYME SAMPLE										
	0.882	0.763	0.119	0.668	0.095	0.598	0.070	0.120	0.108	0.095
	0.910	0.791	0.119	0.679	0.112	0.623	0.056	0.113	0.114	0.098
	0.863	0.714	0.149	0.606	0.108	0.528	0.078	0.148	0.130	0.111
	0.906	0.747	0.159	0.624	0.123	0.555	0.069	0.162	0.140	0.118
TOTAL			0.546		0.438		0.273	0.543	0.492	0.422
AVERAGE			0.137		0.110		0.068	0.136	0.123	0.106
ACTIVITY			3393.750		3075.000		2637.500			
SPRAY-DRIED SAMPLES										
LYSOZYME (1% LYSOZYME)										
	0.854	0.708	0.146	0.597	0.111	0.526	0.071	0.148	0.130	0.112
	0.847	0.696	0.151	0.587	0.109	0.505	0.082	0.149	0.127	0.113
	0.842	0.673	0.169	0.571	0.102	0.521	0.050	0.167	0.150	0.116
	0.871	0.720	0.151	0.622	0.098	0.548	0.074	0.145	0.123	0.108
TOTAL			0.617		0.420		0.277	0.609	0.530	0.449
AVERAGE			0.154		0.105		0.069	0.152	0.133	0.112
ACTIVITY			3806.250		3312.500		2806.250			
% activity of lysozyme			112.155		107.724		106.398			
FOR 1% LYSOZYME + 1%										

B. CYCLODEXTRIN(Spray-dried)										
	0.838	0.754	0.084	0.684	0.070	0.626	0.058	0.084	0.078	0.071
	0.840	0.753	0.087	0.679	0.074	0.624	0.055	0.085	0.083	0.073
	0.836	0.792	0.044	0.723	0.069	0.689	0.034	0.011	0.051	0.050
	0.875	0.833	0.042	0.800	0.033	0.759	0.041	0.043	0.039	0.039
							0.000			
TOTAL			0.257		0.246		0.188	0.223	0.251	0.233
AVERAGE			0.064		0.062		0.047	0.056	0.063	0.058
ACTIVITY			1393.750		1568.750		1456.250			
% activity of lysozyme			41.068		51.016		55.213			
FOR 1% LYSOZYME + 1% B. CYCLODEXTRIN(ElectroSpray- dried)										
	0.859	0.743	0.116	0.649	0.094	0.579	0.070	0.116	0.104	0.093
	0.888	0.780	0.108	0.689	0.091	0.624	0.065	0.109	0.102	0.090
	0.854	0.729	0.125	0.636	0.093	0.560	0.076	0.126	0.107	0.097
	0.830	0.729	0.101	0.617	0.112	0.560	0.057	0.096	0.103	0.094
TOTAL			0.450		0.390		0.268	0.447	0.416	0.374
AVERAGE			0.113		0.098		0.067	0.112	0.104	0.094
ACTIVITY			2793.750		2600.000		2337.500			
% activity of lysozyme			82.320		84.553		88.626			

ELECTRO SPRAY-DRIED SAMPLES										
LYSOZYME (1% LYSOZYME)	0.888	0.796	0.092	0.715	0.081	0.652	0.063	0.090	0.086	0.079
	0.859	0.765	0.094	0.688	0.077	0.62	0.068	0.094	0.086	0.079
	0.912	0.792	0.120	0.693	0.099	0.62	0.073	0.118	0.108	0.098
	0.839	0.699	0.140	0.596	0.103	0.523	0.073	0.142	0.125	0.106
TOTAL			0.446		0.360		0.277	0.444	0.405	0.362
AVERAGE			0.112		0.090		0.069	0.111	0.101	0.091
ACTIVITY			2775.000		2531.250		2262.500			
% activity of lysozyme			81.768		82.317		85.782			

BIOLOGICAL ACTIVITY OF SPRAY-DRIED AND ELECTROSPRAYED LYSOZYME SAMPLE							
TIME	**0 MIN**	**1 MIN**		**2 MINS**		**3 MINS**	
BLANK	1.439	1.430	0.009	1.433	(0.003)	1.432	0.001
1% UNPROCESSED LYSOZYME							
	0.804	0.353	0.451	0.224	0.129	0.165	0.059
	0.607	0.258	0.349	0.176	0.082	0.143	0.033
	0.799	0.433	0.366	0.259	0.174	0.184	0.075
TOTAL			**1.166**		**0.385**		**0.167**

AVERAGE			0.389		0.128		0.056
ACTIVITY UNIT/MG			863.704		(855.556)		1113.333
1% SPRAY-DRIED LYSOZYME							
	0.738	0.209	0.529	0.161	0.048	0.136	0.025
	0.649	0.252	0.397	0.169	0.083	0.138	0.031
	0.762	0.380	0.382	0.165	0.215	0.136	0.029
TOTAL			**1.308**		**0.346**		**0.085**
AVERAGE			**0.436**		**0.115**		**0.028**
ACTIVITY UNIT/MG			968.889		(768.889)		566.667
% ACTIVITY			112.178		89.870		50.898
1:1 % SPRAY-DRIED LYSOZYME, BETACYCLODEXTRIN							
	0.609	0.253	0.356	0.172	0.081	0.141	0.031
	0.638	0.298	0.340	0.188	0.110	0.142	0.046
	0.726	0.340	0.386	0.197	0.143	0.148	0.049
TOTAL			**1.082**		**0.334**		0.126
AVERAGE			0.361		0.111		0.042
ACTIVITY UNIT/MG			801.481		(742.222)		840.000
% ACTIVITY			92.796		86.753		75.449
1:1:1 % SPRAY-DRIED LYSOZYME, BETACYCLODEXTRIN, PLURONIC							
	0.766	0.412	0.354	0.267	0.145	0.208	0.059
	0.877	0.470	0.407	0.279	0.191	0.200	0.079
	0.720	0.363	0.357	0.244	0.119	0.175	0.069
TOTAL			1.118		**0.455**		**0.207**
AVERAGE			**0.373**		**0.152**		**0.069**
ACTIVITY UNIT/MG			828.148		(1011.111)		1380.667
% ACTIVITY			**95.883**		118.182		**124.012**
1:1% SPRAY-DRIED LYSOZYME, PLURONIC							
	0.481	0.214	0.267	0.169	0.045	0.143	0.026
	0.388	0.218	0.170	0.174	0.044	0.148	0.026

	0.612	0.236	0.376	0.181	0.055	0.152	0.029
TOTAL			0.813		0.144		0.081
AVERAGE			**0.271**		**0.048**		**0.027**
ACTIVITY UNIT/MG			602.222		(320.000)		540.000
% ACTIVITY			**69.726**		**37.403**		**48.503**
1% ELECTRO-SPRAYED LYSOZYME							
	0.750	0.437	0.313	0.286	0.151	0.210	0.076
	0.838	0.457	0.381	0.312	0.145	0.247	0.065
	0.776	0.375	0.401	0.232	0.143	0.185	0.047
TOTAL			1.095		0.439		0.188
AVERAGE			**0.365**		**0.146**		**0.063**
ACTIVITY UNIT/MG			811.111		(975.556)		1253.333
% ACTIVITY			**93.911**		**114.026**		**112.575**
FOR 1% ELECTRO-SPRAYED LYSOZYME + 1% BCYO + 1% PLURONIC							
	0.681	0.280	0.401	0.178	0.102	0.143	0.035
	0.745	0.339	0.406	0.206	0.133	0.149	0.057
	0.749	0.352	0.397	0.208	0.144	0.147	0.061
TOTAL			1.204		0.379		0.153
AVERAGE			0.401		0.126		0.051
ACTIVITY UNIT/MG			891.852		(842.222)		1020.000
% ACTIVITY			**103.259**		**98.442**		**91.617**
1% ELECTRO-SPRAYED LYSOZYME + 1% BETA CYCLODEXTRIN							
	0.695	0.297	0.398	0.187	0.110	0.148	0.039
	0.698	0.357	0.341	0.220	0.137	0.161	0.059
	0.730	0.349	0.381	0.212	0.137	0.161	0.051
TOTAL			1.120		0.384		0.149
AVERAGE			0.373		0.128		0.050
ACTIVITY UNIT/MG			829.630		(853.333)		993.333
% ACTIVITY			**96.055**		**99.740**		**89.222**

FOR 1% ELECTRO-SPRAYED LYSOZYME + 1% PLURONIC							
	0.650	0.284	0.366	0.193	0.091	0.153	0.040
	0.755	0.443	0.312	0.284	0.159	0.188	0.096
	0.708	0.318	0.390	0.213	0.105	0.163	0.050
TOTAL			1.068		0.355		0.186
AVERAGE			0.356		0.118		0.062
ACTIVITY UNIT/MG			791		-789		1240
% ACTIVITY			**91.60**		**92.21**		**111.38**

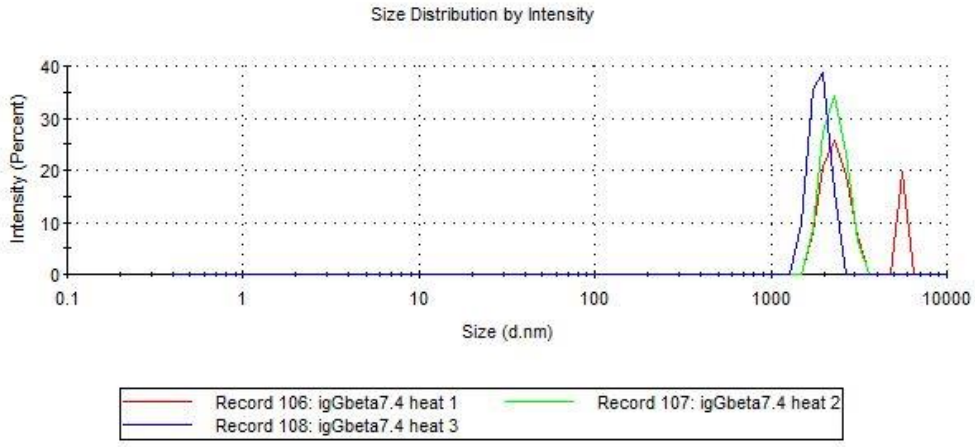
Appendices vi: **Particle Size for Lysozyme Formulation**

Sample Name: igGbeta7.4 heat 1
SOP Name: mansettings.nano
File Name: monoclonal antibody.dts
Record Number: 106
Material RI: 1.45
Material Absorbtion: 0.001
Dispersant Name: sodium citrate
Dispersant RI: 1.580
Viscosity (cP): 1.3300
Measurement Date and Time: 26 November 2020 16:4

Temperature (°C): 25.0
Count Rate (kcps): 217.6
Cell Description: Disposable sizing cuvette
Duration Used (s): 70
Measurement Position (mm): 1.25
Attenuator: 4

			Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm):	5864	Peak 1:	2325	80.4	382.3
PdI:	0.274	Peak 2:	5560	19.6	6.104e-5
Intercept:	0.923	Peak 3:	0.000	0.0	0.000

Result quality : Refer to quality report



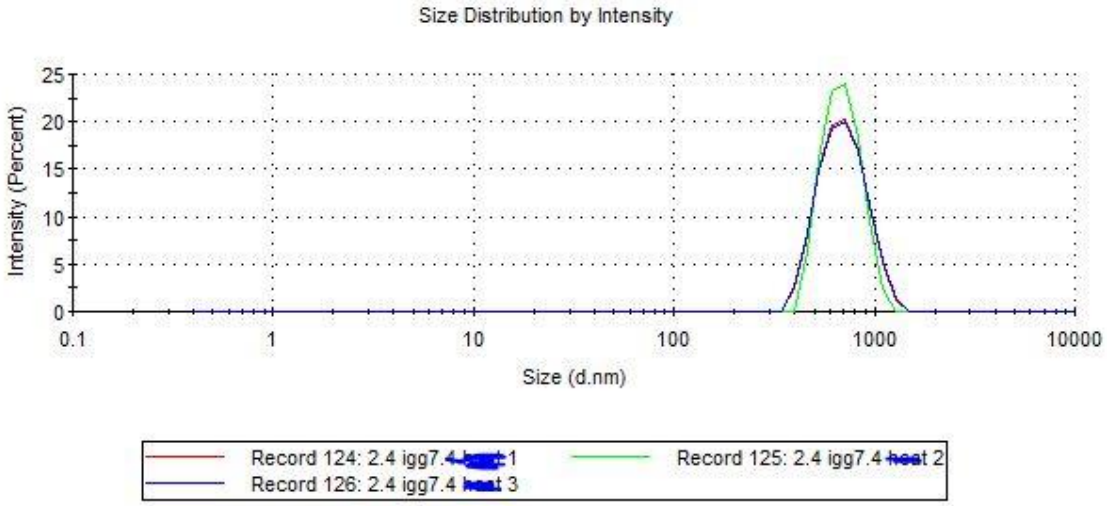
Tab le:

Showing the Particle Size Analysis

Sample Name:	2.4 igg7.4 heat 1		
SOP Name:	size measurement.sop		
File Name:	monoclonal antibody.dts	**Dispersant Name:**	Water
Record Number:	124	**Dispersant RI:**	1.330
Material RI:	1.52	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.000	**Measurement Date and Time:**	31 January 2022 18:02:30

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	396.1	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	8

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	650.5	**Peak 1:**	711.0	100.0	187.0
PdI:	0.253	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.942	**Peak 3:**	0.000	0.0	0.000
Result quality :	Good				



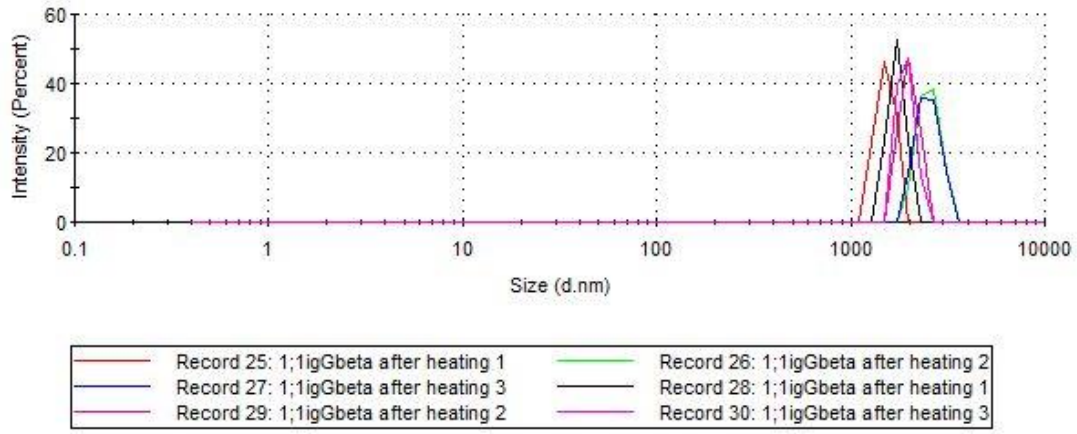
Sample Name:	1;1igGbeta after heating 1		
SOP Name:	mansettings.nano		
File Name:	monoclonal antibody.dts	**Dispersant Name:**	sodium citrate
Record Number:	25	**Dispersant RI:**	1.580
Material RI:	1.45	**Viscosity (cP):**	1.3300
Material Absorbtion:	0.001	**Measurement Date and Time:**	15 October 2020 14:14:35

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	396.3	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	7

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	3153	**Peak 1:**	1509	100.0	160.4
PdI:	1.000	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.941	**Peak 3:**	0.000	0.0	0.000

Result quality : Refer to quality report

Size Distribution by Intensity

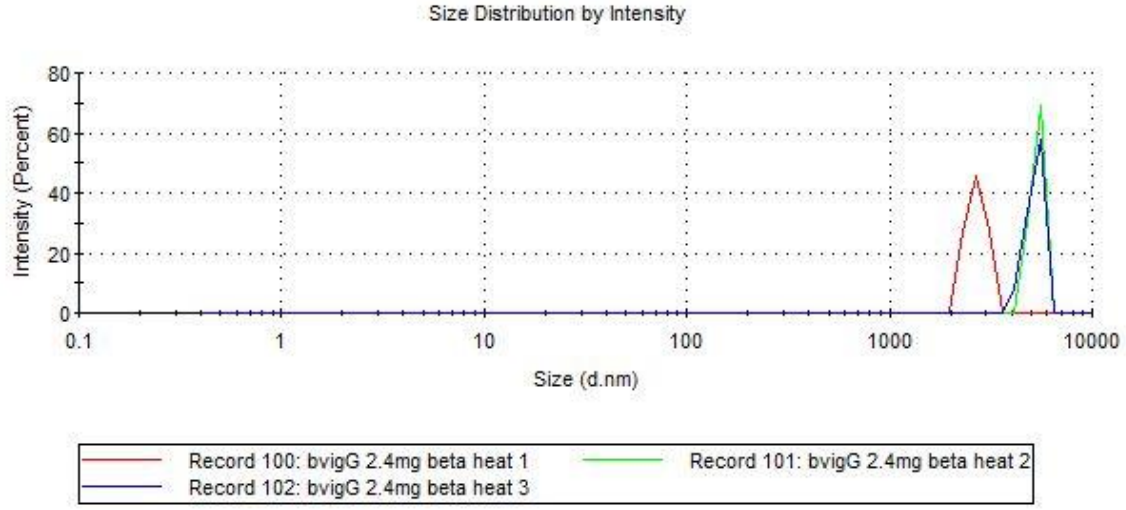


Sample Name:	bvigG 2.4mg beta heat 1		
SOP Name:	mansettings.nano		
File Name:	monoclonal antibody.dts	**Dispersant Name:**	sodium citrate
Record Number:	100	**Dispersant RI:**	1.580
Material RI:	1.45	**Viscosity (cP):**	1.3300
Material Absorbtion:	0.001	**Measurement Date and Time:**	18 November 2020 17:

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	523.4	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	10

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	9143	**Peak 1:**	2689	100.0	289.9
PdI:	1.000	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.435	**Peak 3:**	0.000	0.0	0.000

Result quality : Refer to quality report

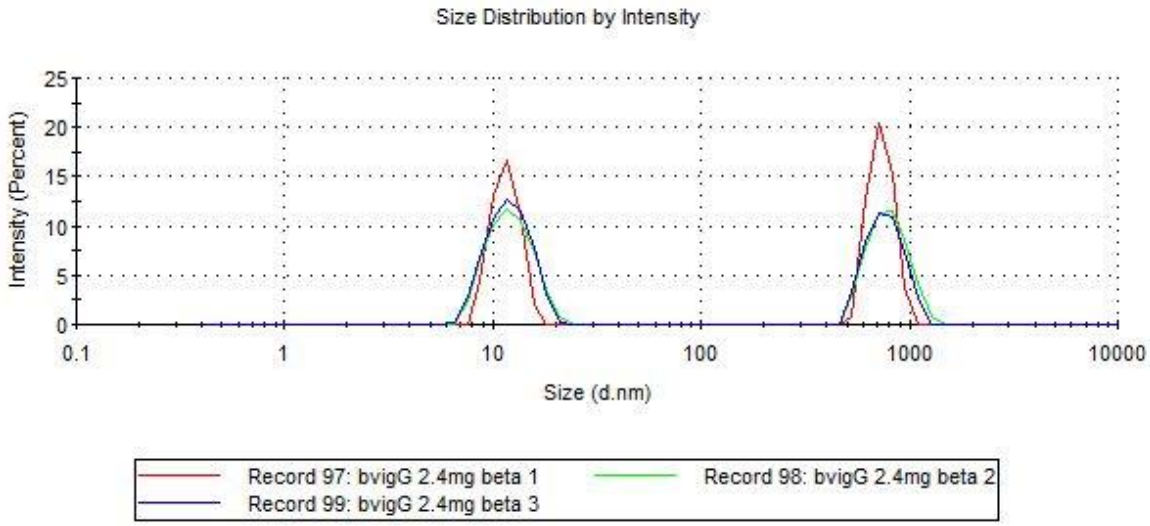


Sample Name:	bvigG 2.4mg beta 1		
SOP Name:	mansettings.nano		
File Name:	monoclonal antibody.dts	**Dispersant Name:**	sodium citrate
Record Number:	97	**Dispersant RI:**	1.580
Material RI:	1.45	**Viscosity (cP):**	1.3300
Material Absorbtion:	0.001	**Measurement Date and Time:**	18 November 2020 1

Temperature (°C):	25.0	**Duration Used (s):**	80
Count Rate (kcps):	166.4	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	9

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	452.5	**Peak 1:**	736.6	53.0	100.6
PdI:	0.641	**Peak 2:**	11.58	47.0	1.724
Intercept:	0.951	**Peak 3:**	0.000	0.0	0.000

Result quality : Refer to quality report

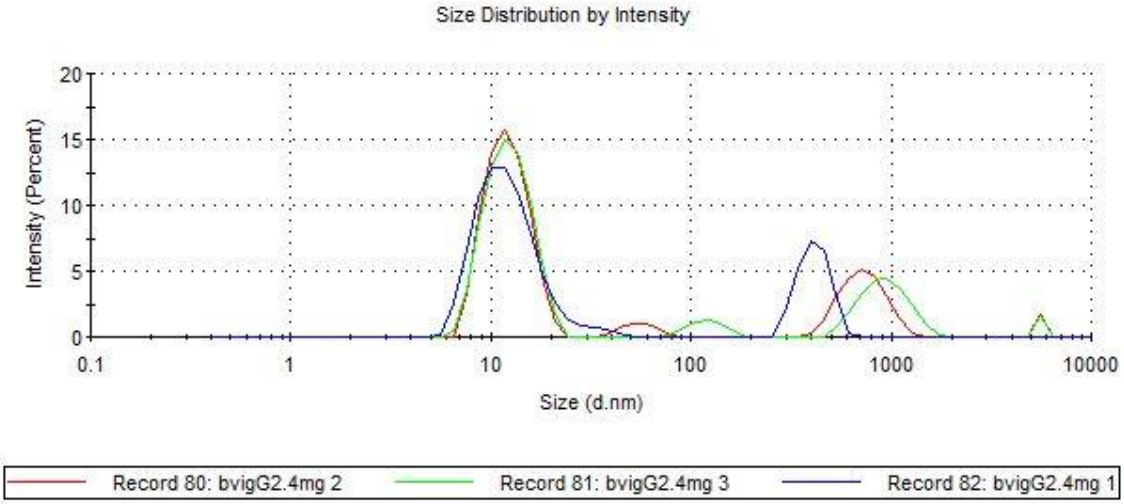


Sample Name:	bvigG2.4mg 2		
SOP Name:	mansettings.nano		
File Name:	monoclonal antibody.dts	**Dispersant Name:**	sodium citrate
Record Number:	80	**Dispersant RI:**	1.580
Material RI:	1.45	**Viscosity (cP):**	1.3300
Material Absorbtion:	0.001	**Measurement Date and Time:**	17 November 2020 15:(

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	243.1	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	8

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	109.9	**Peak 1:**	12.28	70.8	3.004
PdI:	0.361	**Peak 2:**	737.6	23.6	182.2
Intercept:	0.943	**Peak 3:**	55.25	3.9	10.15

Result quality : Refer to quality report



Appendix vii: SEM for lysozyme formulation.

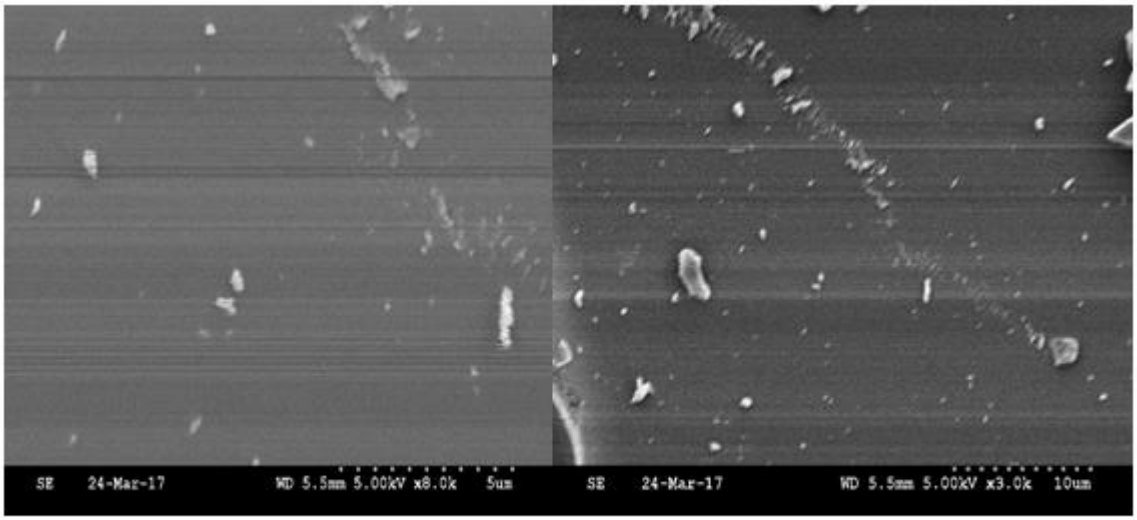


Figure: Unprocessed Lysozyme (without Excipients)

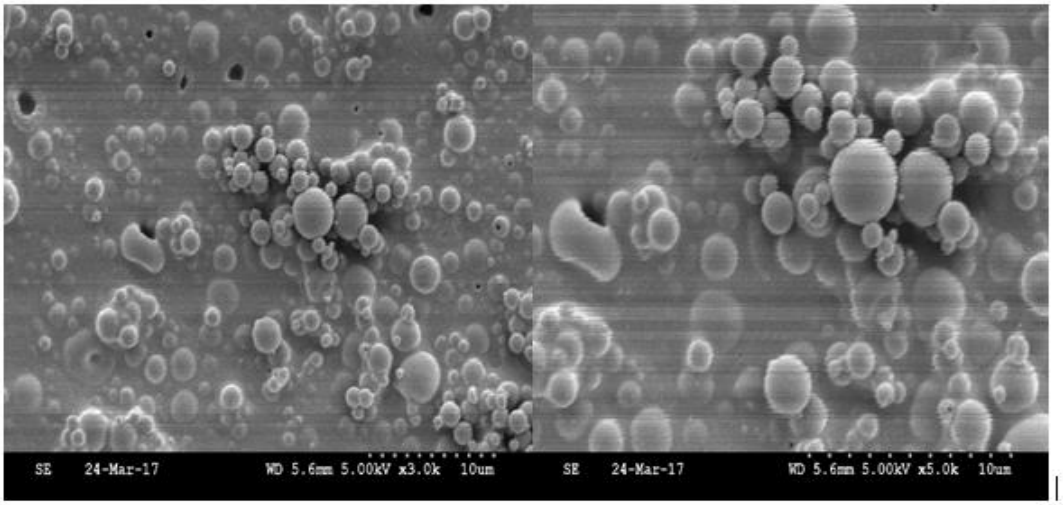


Figure : 1% Spraydried Lysozyme

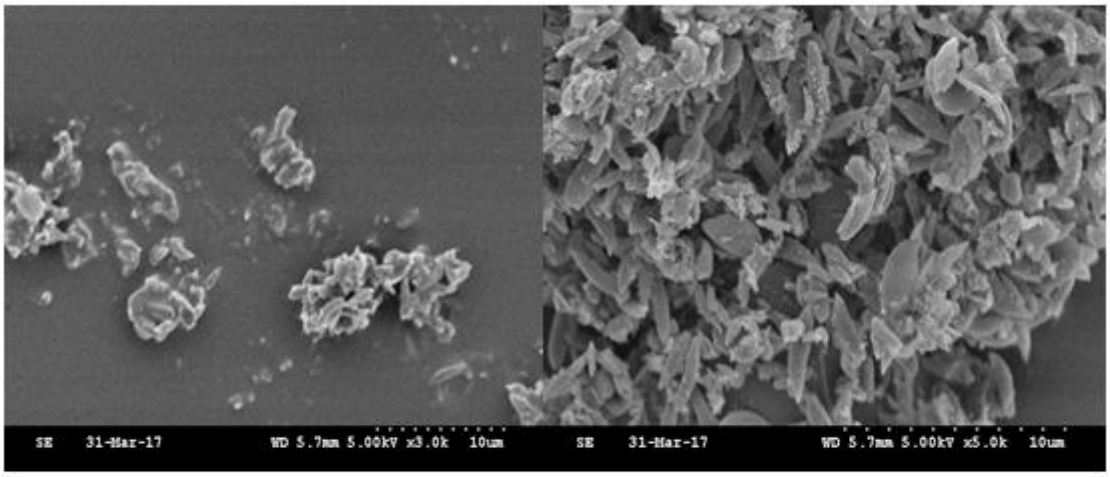


Figure: 1% Electrosprayed Dried Lysozyme

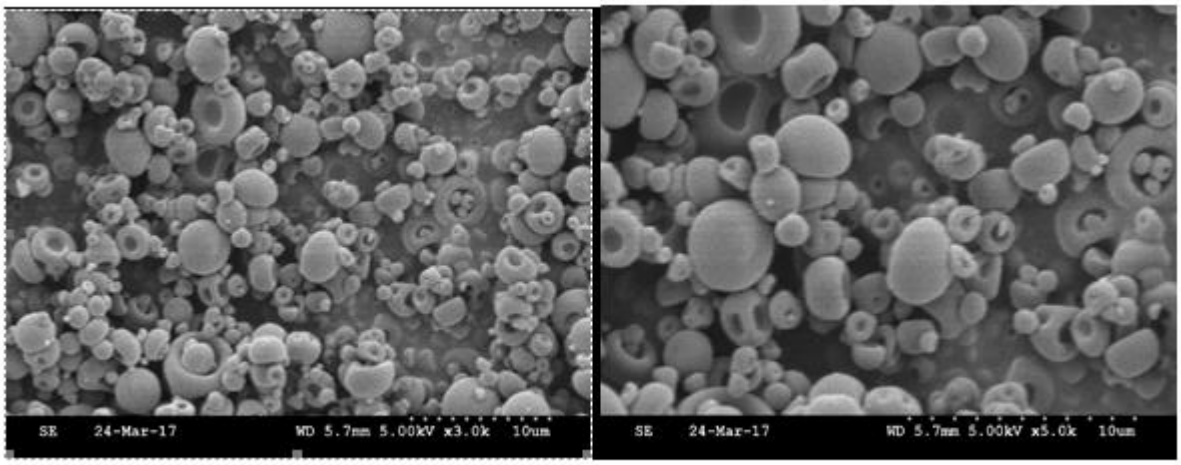


Figure: 1% Spraydried Lysozyme with 1% Betacyclodextrin

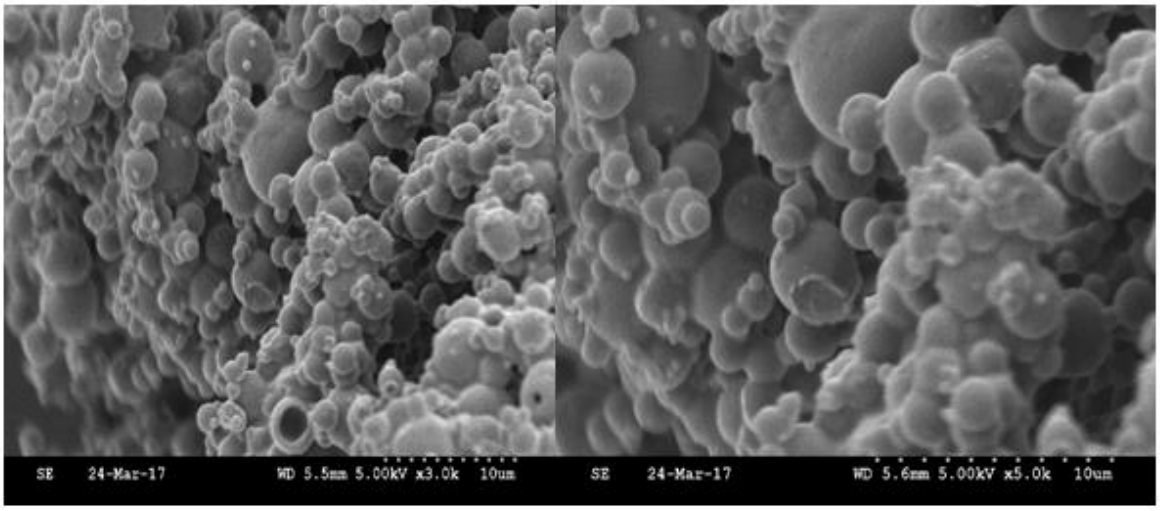


Figure: 1% Spraydried Lysozyme with 1% Betacyclodextrin and 5% Pluronic

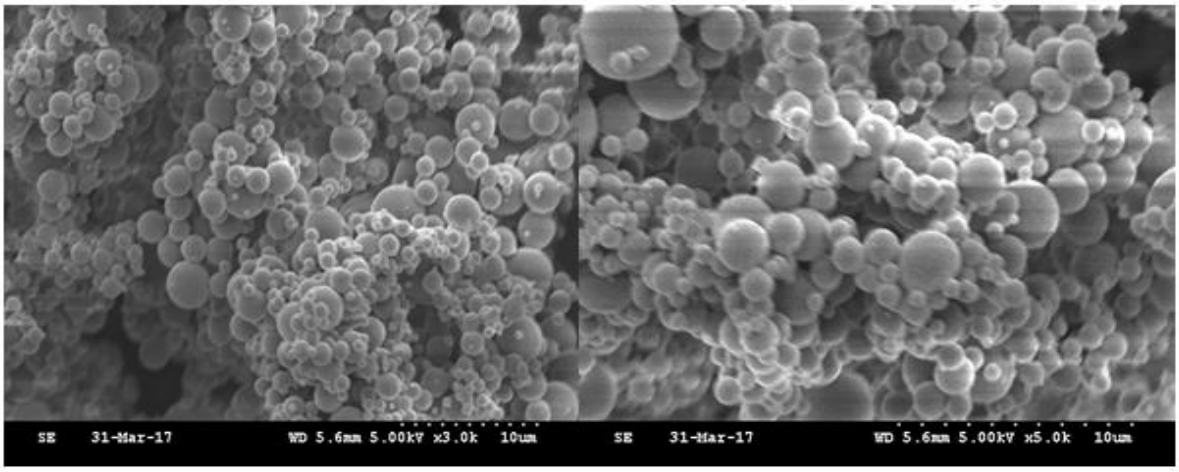


Figure: 1% Spraydried Lysozyme with 5% Pluronic

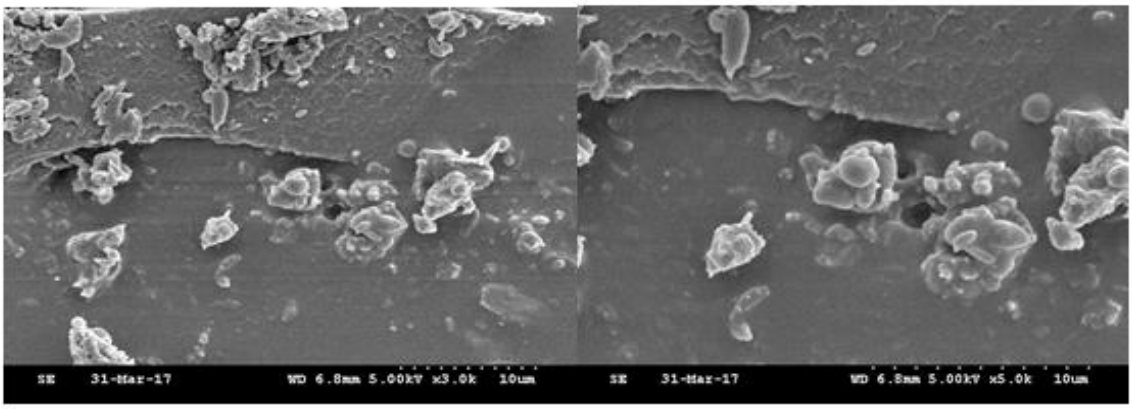


Figure: 1% Electrosprayed dried Lysozyme with 1% Betacyclodextrin

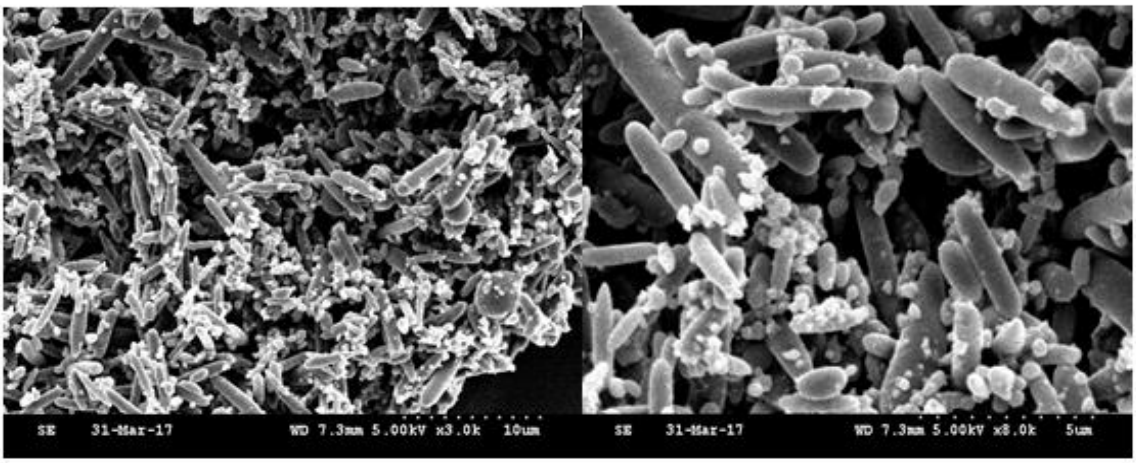


Figure: 1% Electrosprayed dried Lysozyme with 1%Pluronic

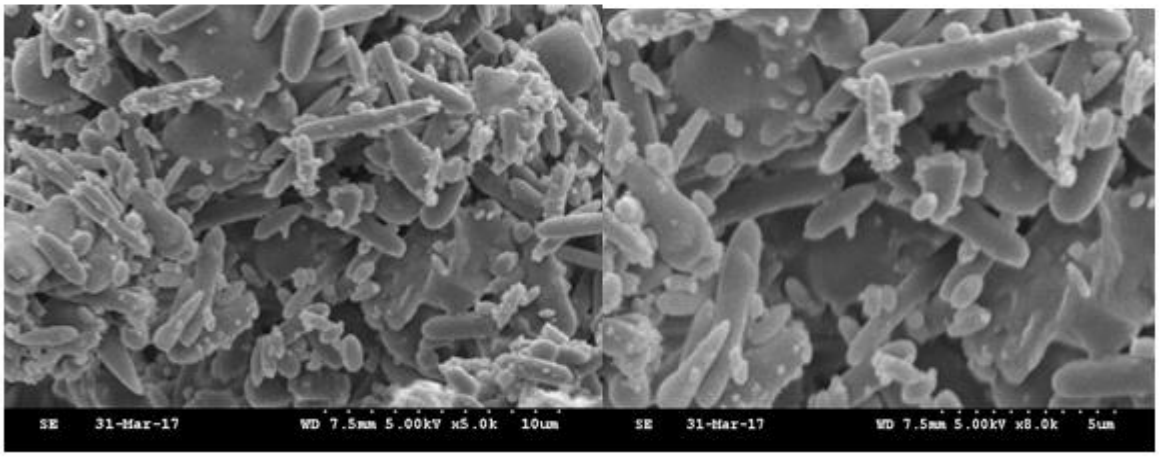
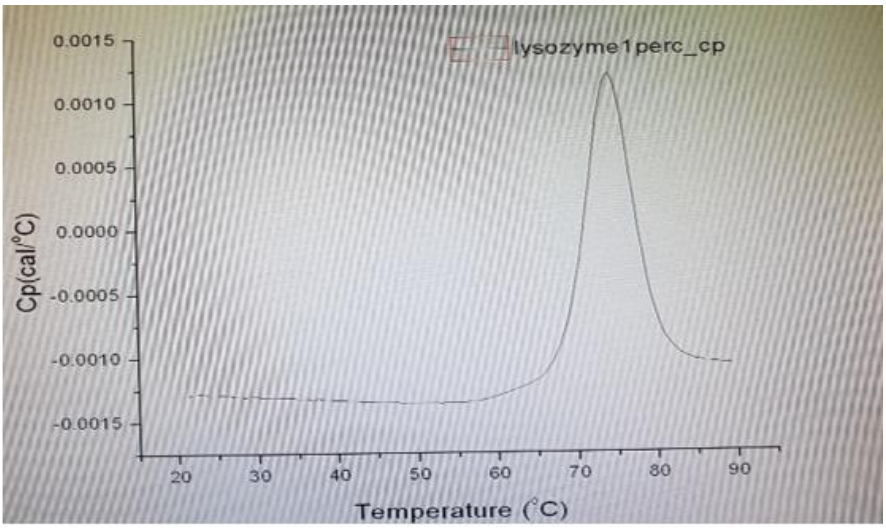
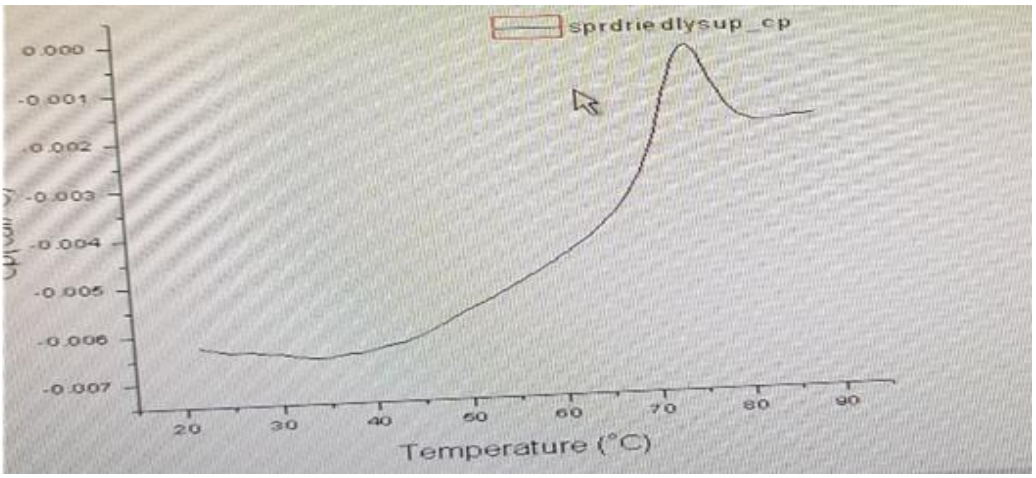
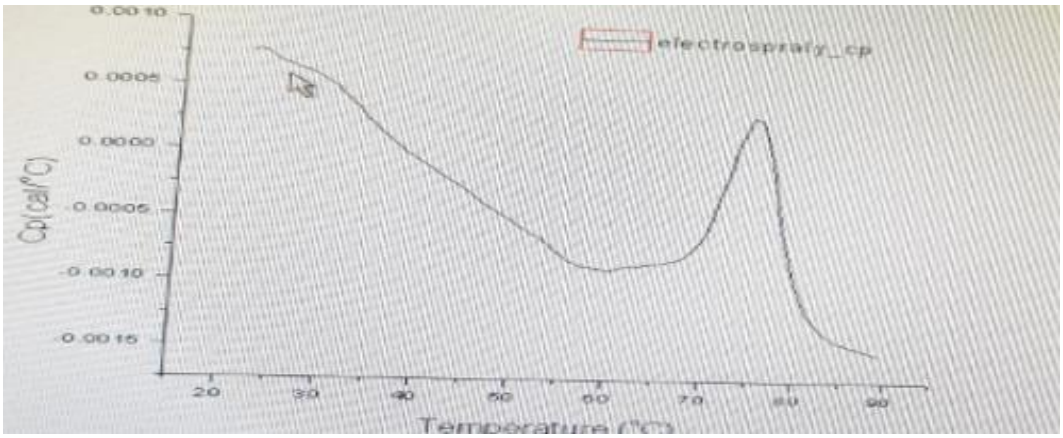


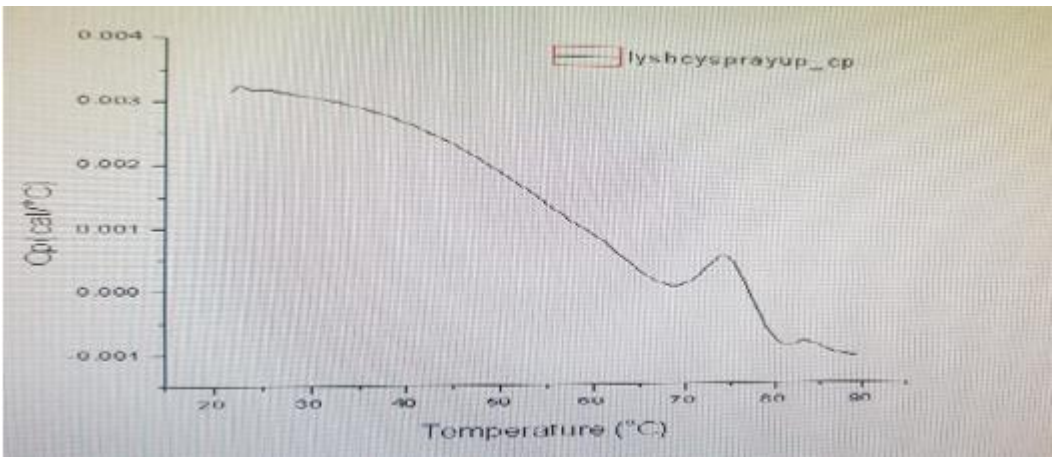
Figure: 1% Electrosprayed Lysozyme with 1% Betacyclodextrin and 1% Pluronic

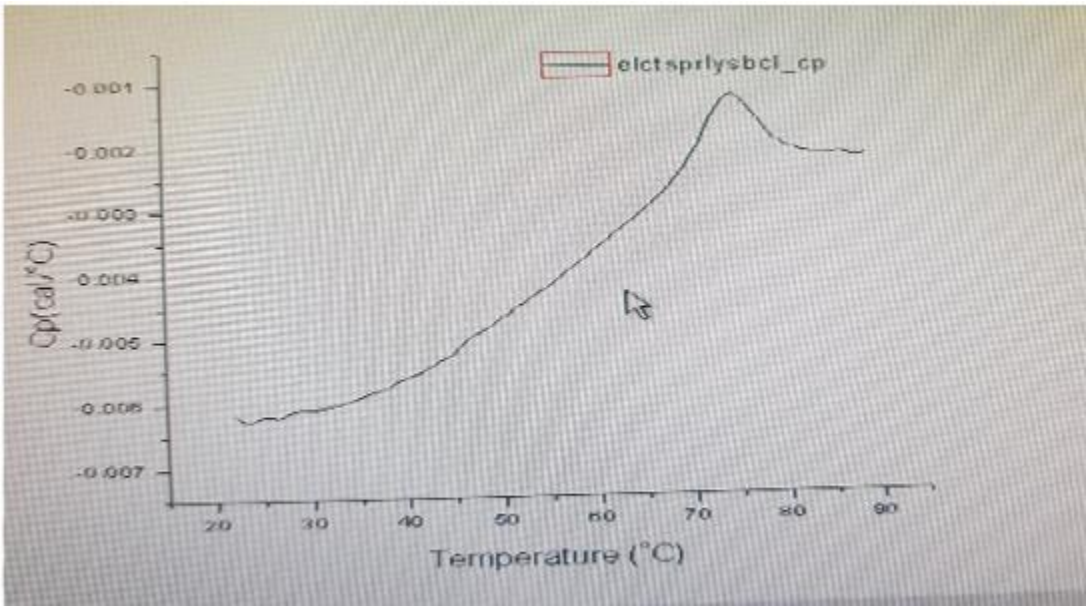
Appendix viii: Hsdsc data for lysozyme formulation

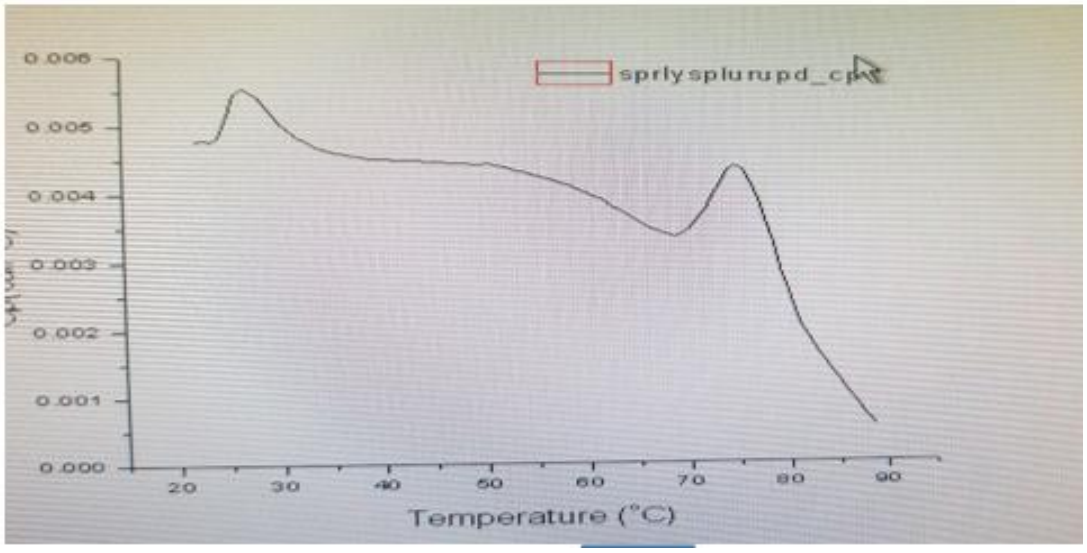


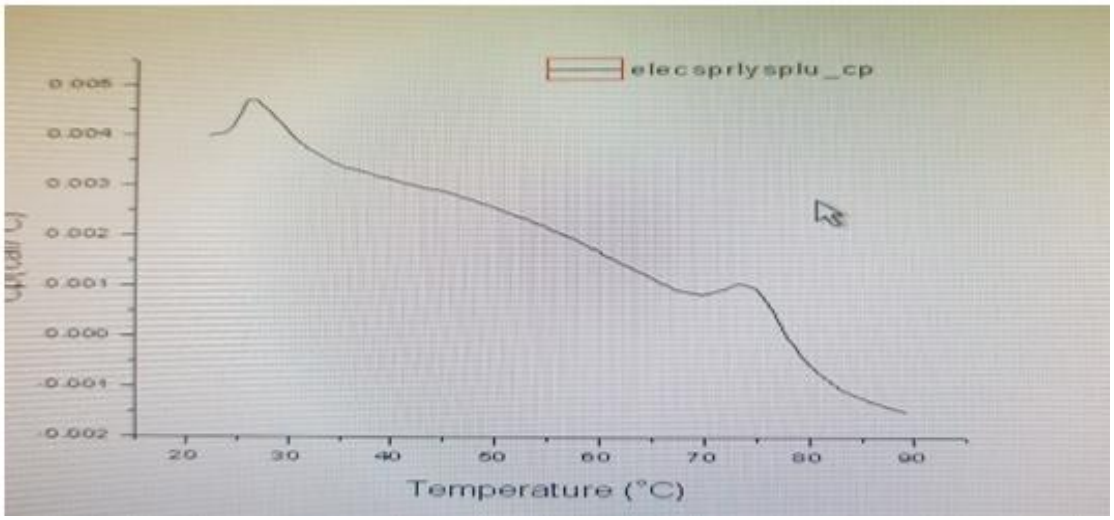


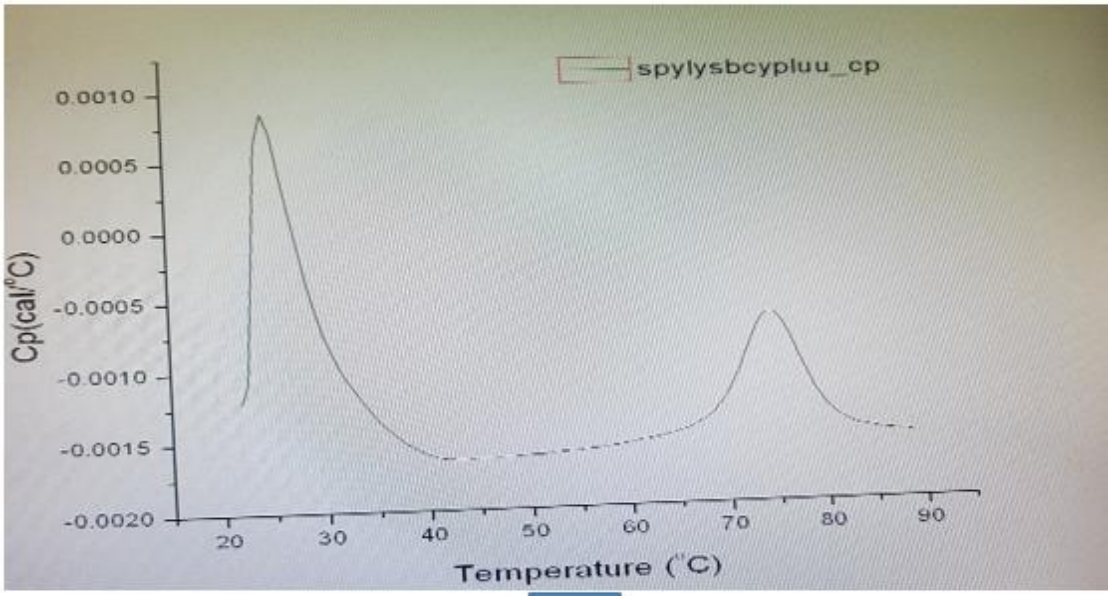


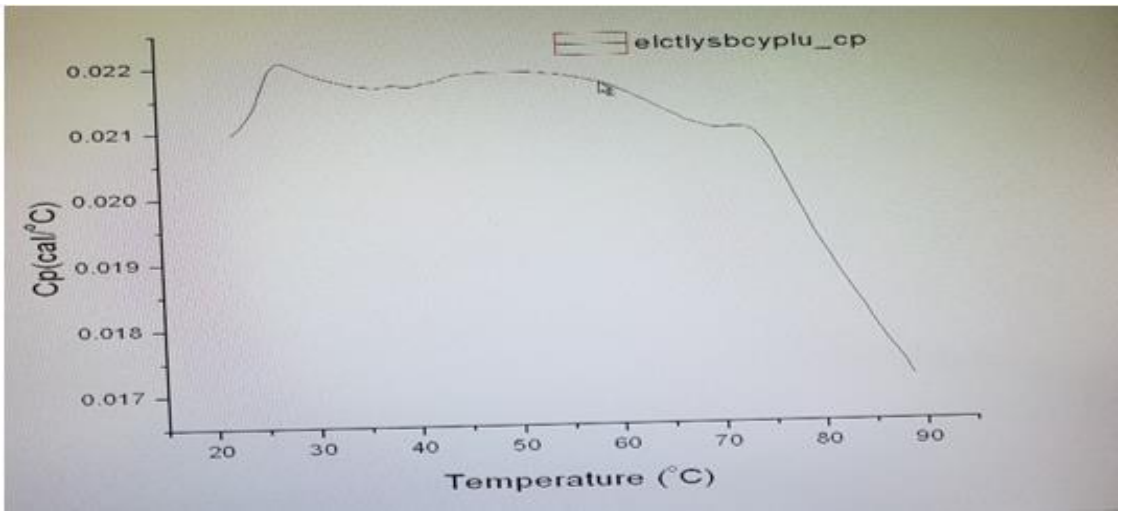

lysbcysprayup_cp
Cp(cal/°C)
0.004
0.003
0.002
0.001
0.000
-0.001
20
30
40
50
60
70
80
90
Temperature (°C)


elctsprlysbcl_cp
Cp(cal/°C)
-0.001
-0.002
-0.003
-0.004
-0.005
-0.006
-0.007
20
30
40
50
60
70
80
90
Temperature (°C)

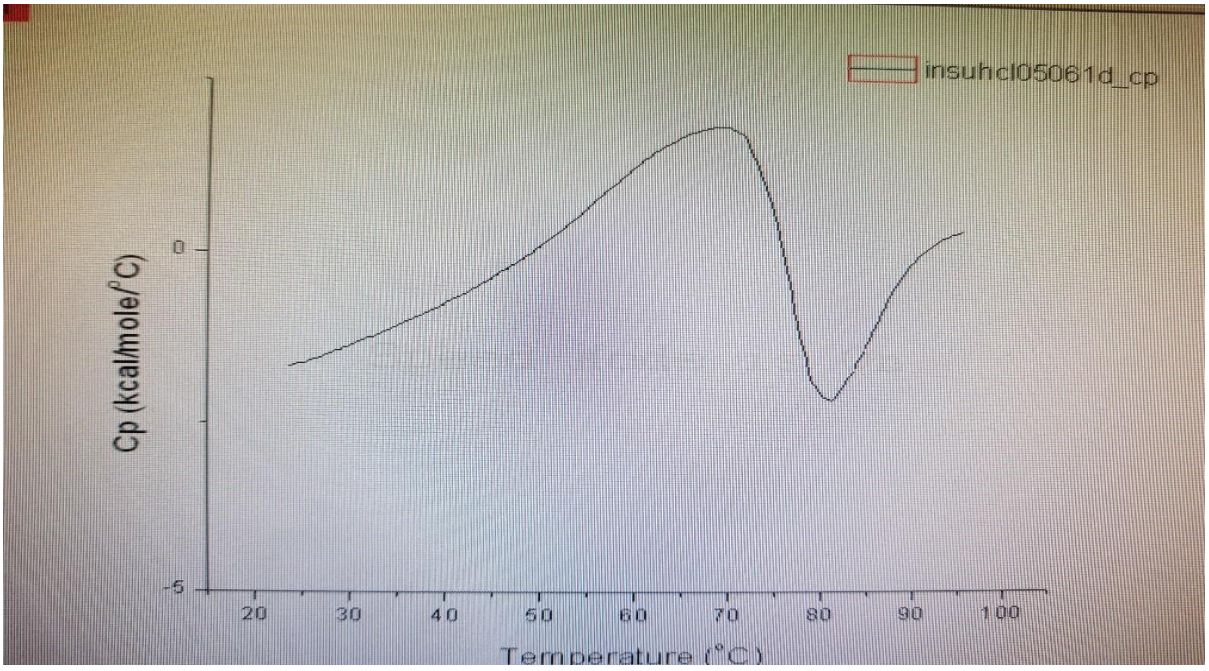

sprlysplunupd_cp
0.006
0.005
0.004
0.003
0.002
0.001
0.000
20
30
40
50
60
70
80
90
Temperature (°C)

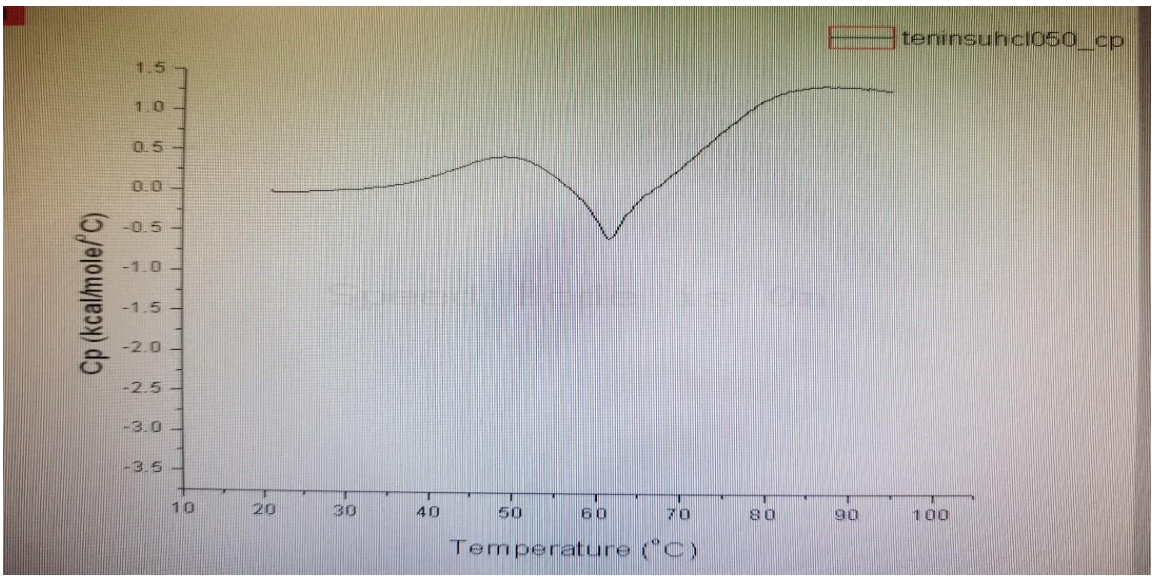

elecsprlysplu_cp
0.005
0.004
0.003
0.002
0.001
0.000
-0.001
-0.002
20
30
40
50
60
70
80
90
Temperature (°C)

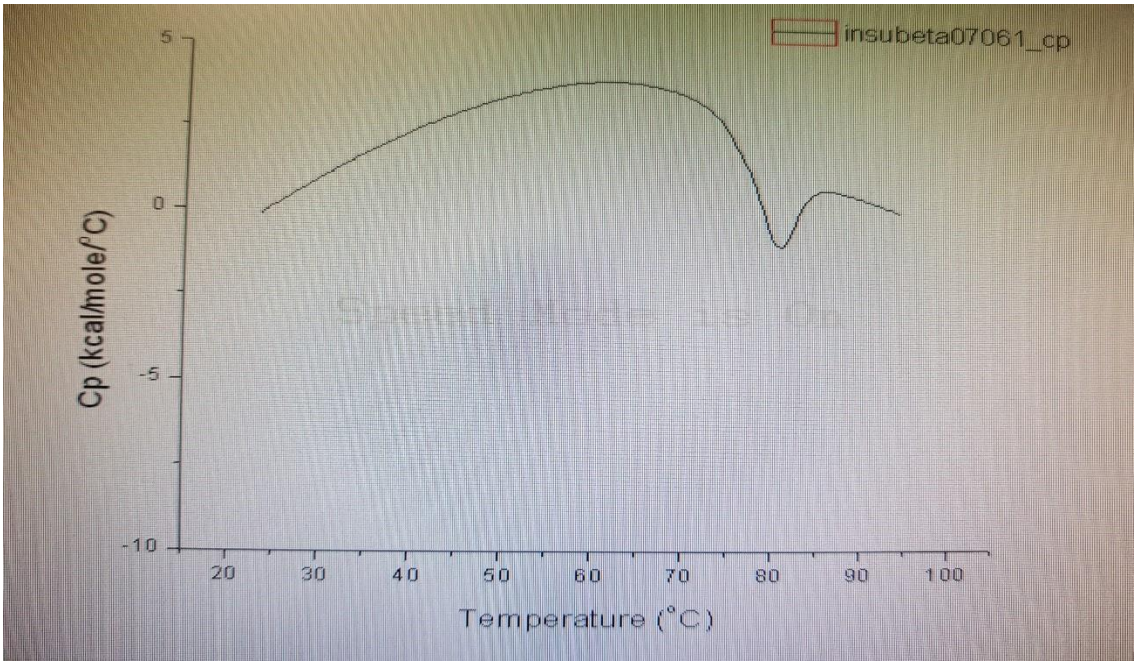

spylysbcypluu_cp
0.0010
0.0005
0.0000
-0.0005
-0.0010
-0.0015
-0.0020
Cp(cal/°C)
20
30
40
50
60
70
80
90
Temperature (°C)

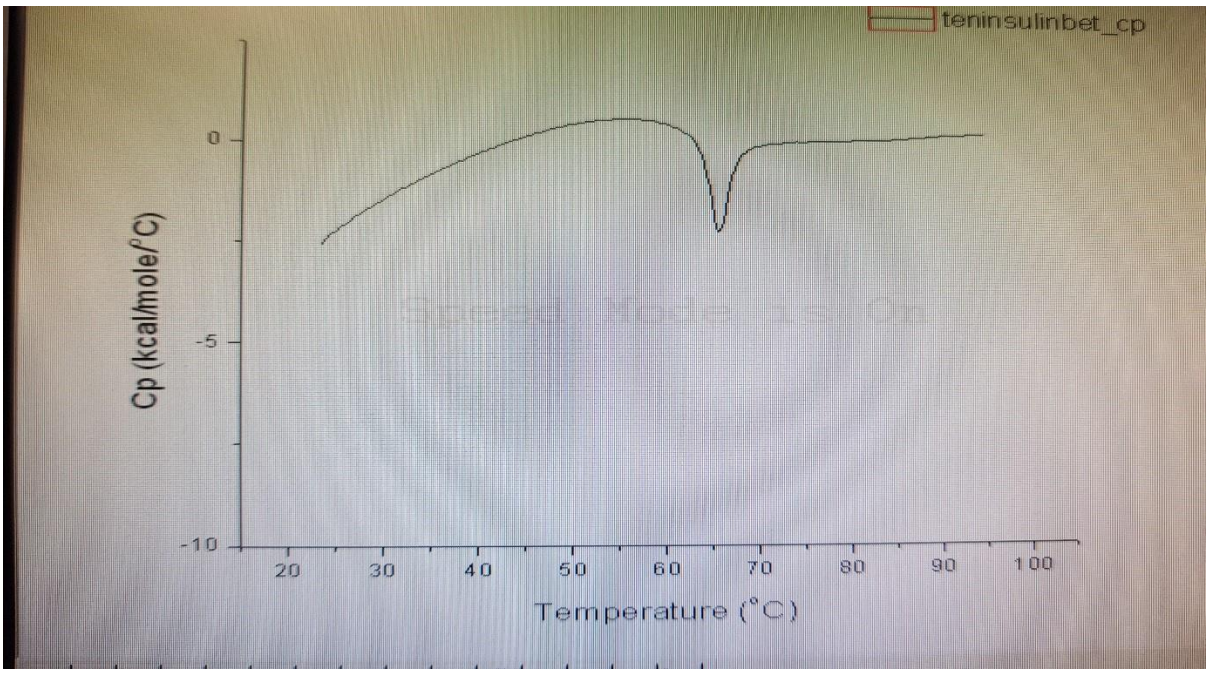

elctlysbcyplu_cp
0.022
0.021
0.020
0.019
0.018
0.017
Cp(cal/°C)
20
30
40
50
60
70
80
90
Temperature (°C)

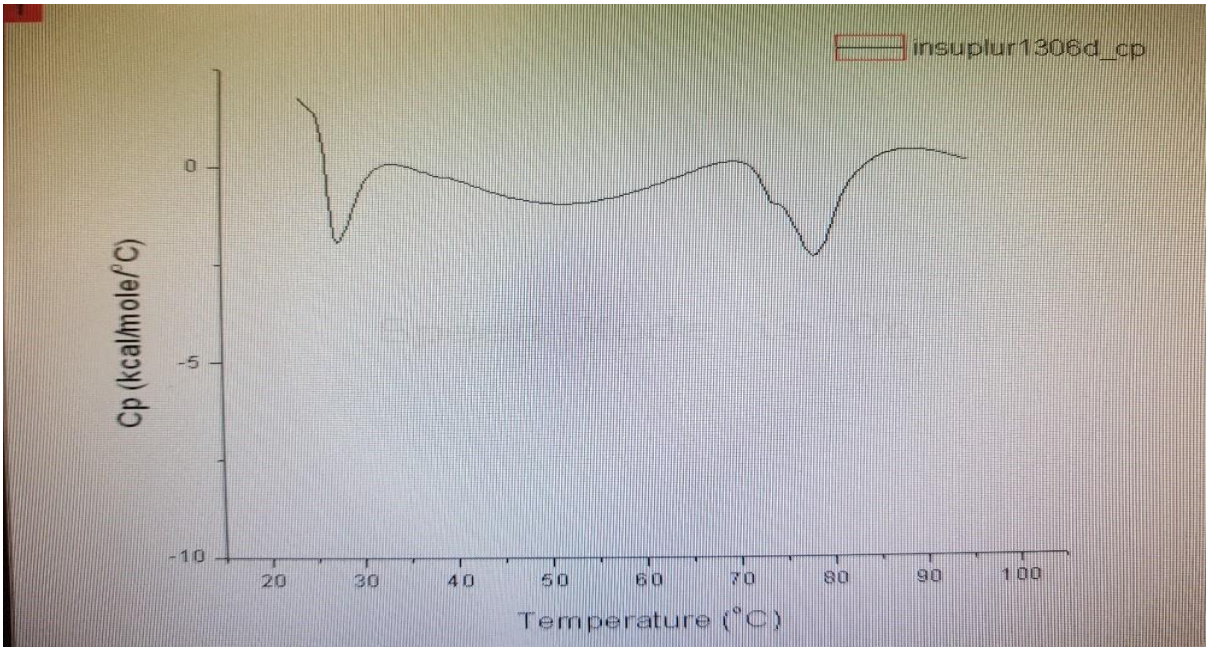
Appendix ix: **Insulin Thermograms**

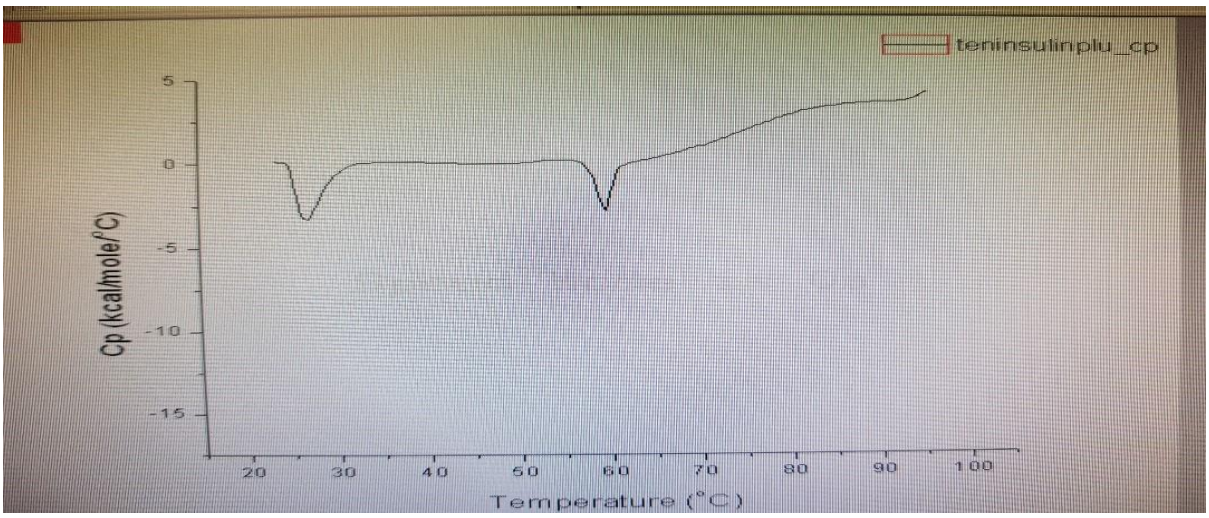


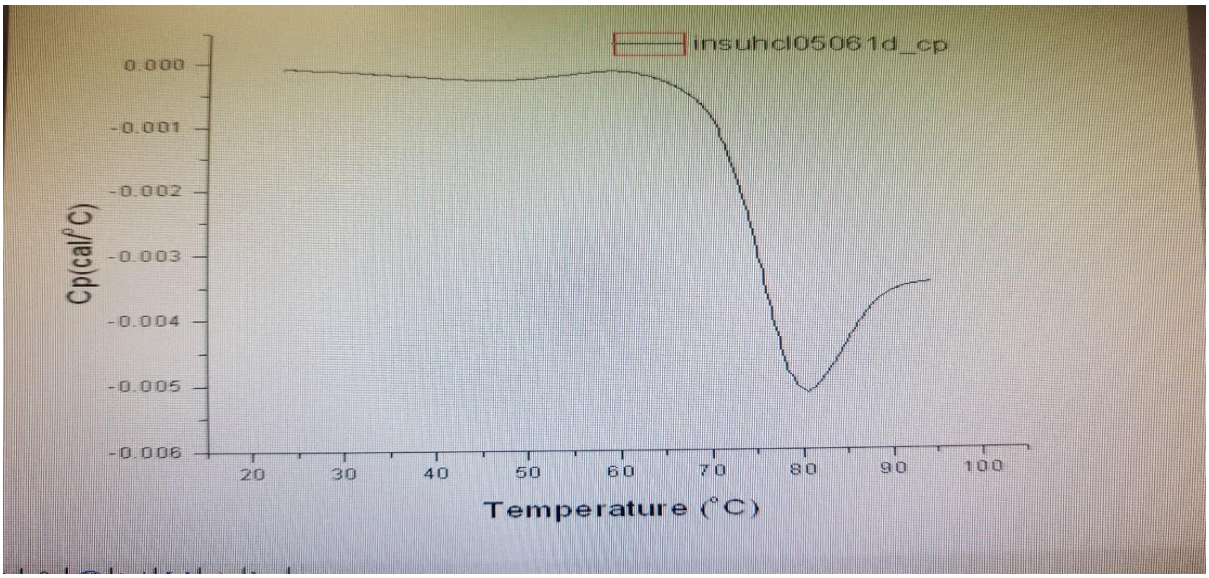

teninsuhcl050_cp
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
Cp (kcal/mole/°C)
10
20
30
40
50
60
70
80
90
100
Temperature (°C)

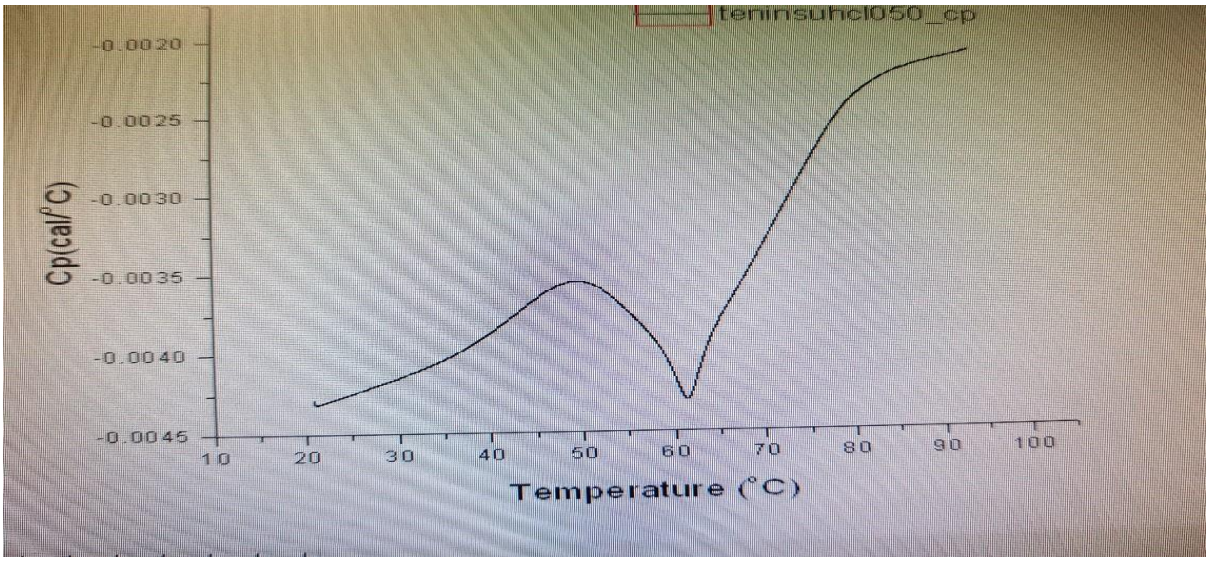

insubeta07061_cp
5
0
-5
-10
Cp (kcal/mole/°C)
20
30
40
50
60
70
80
90
100
Temperature (°C)

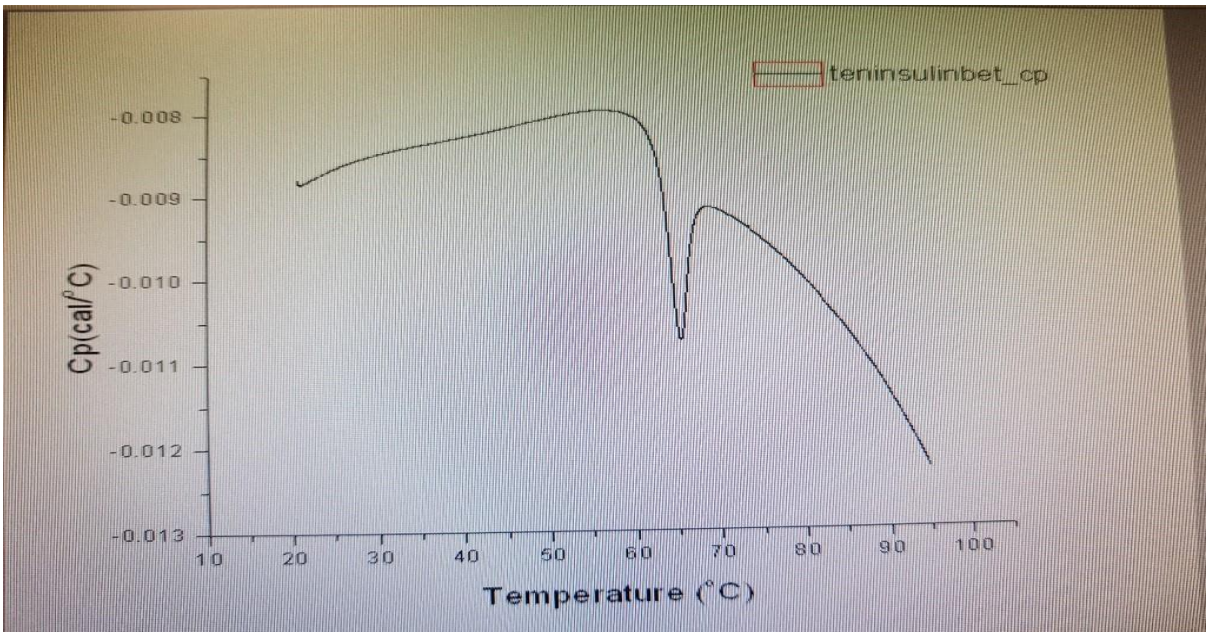

teninsulinbet_cp
Cp (kcal/mole/°C)
0
-5
-10
20
30
40
50
60
70
80
90
100
Temperature (°C)

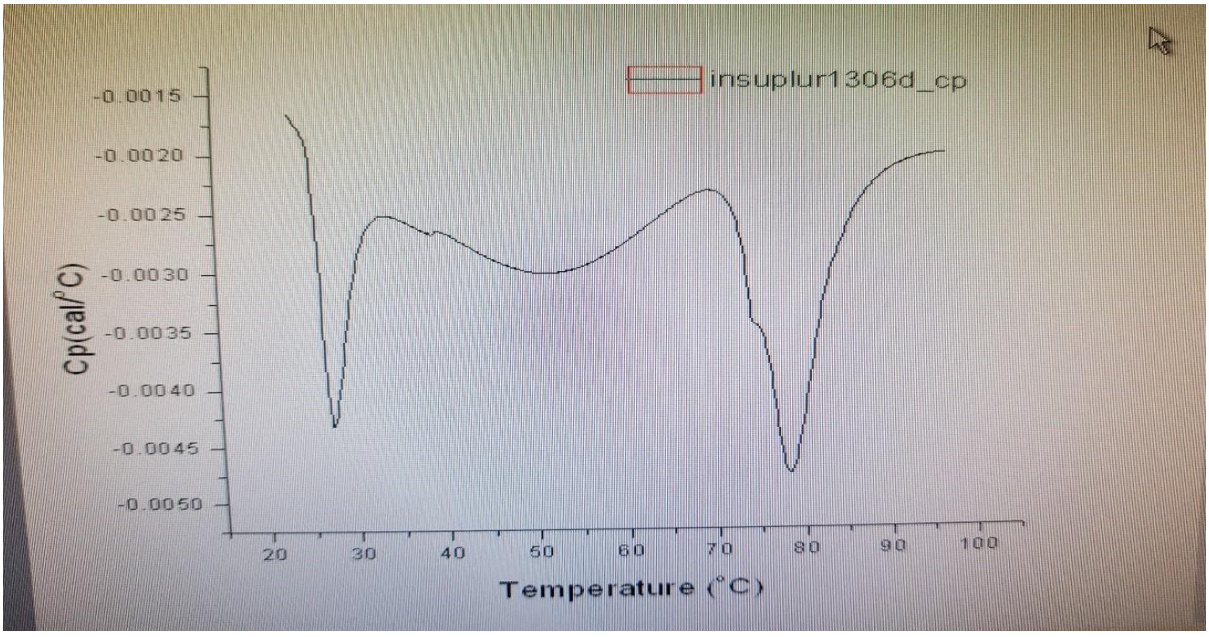

insuplur1306d_cp
Cp (kcal/mole/°C)
0
-5
-10
20
30
40
50
60
70
80
90
100
Temperature (°C)

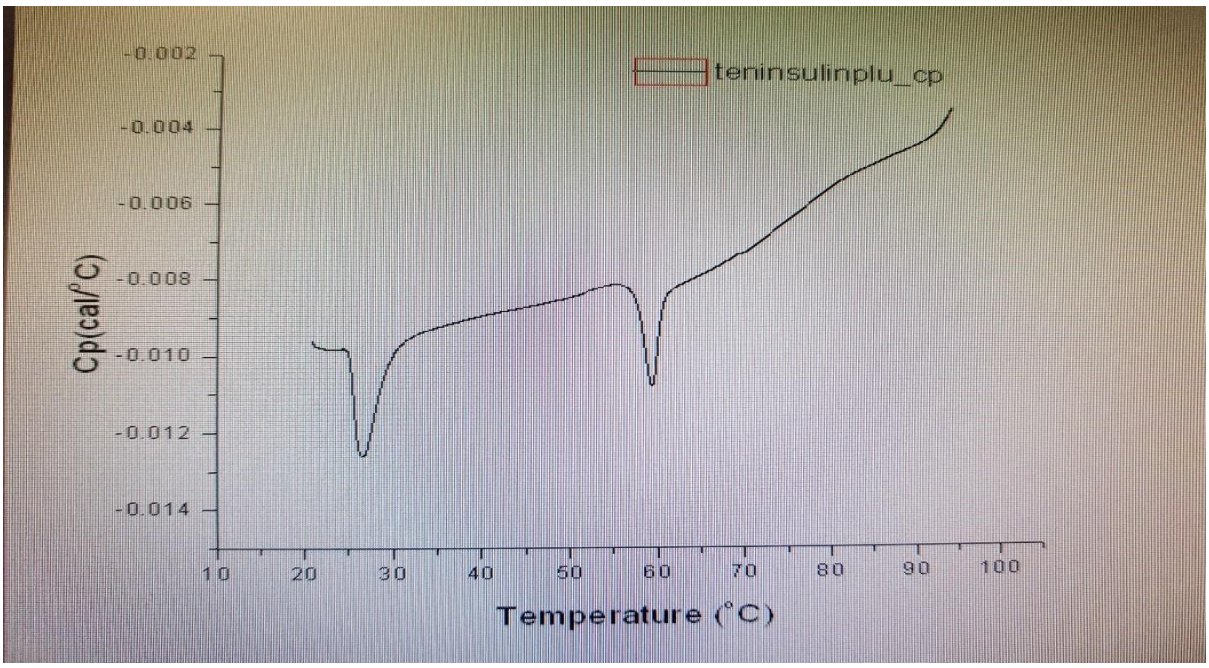

teninsulinplu_cp
Cp (kcal/mole/°C)
5
0
-5
-10
-15
20
30
40
50
60
70
80
90
100
Temperature (°C)

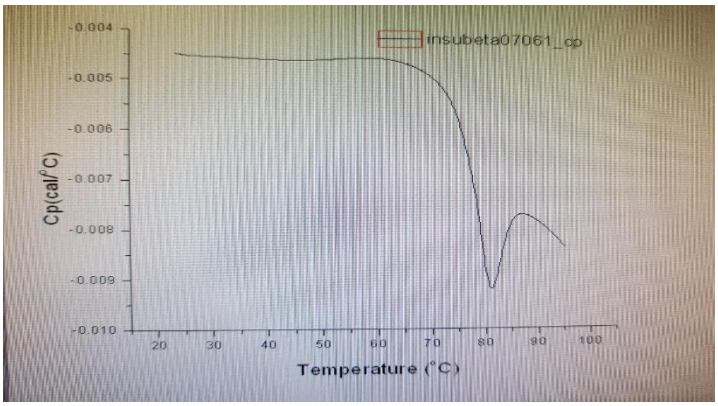

insuhcl05061d_cp
Cp(cal/°C)
0.000
-0.001
-0.002
-0.003
-0.004
-0.005
-0.006
20
30
40
50
60
70
80
90
100
Temperature (°C)

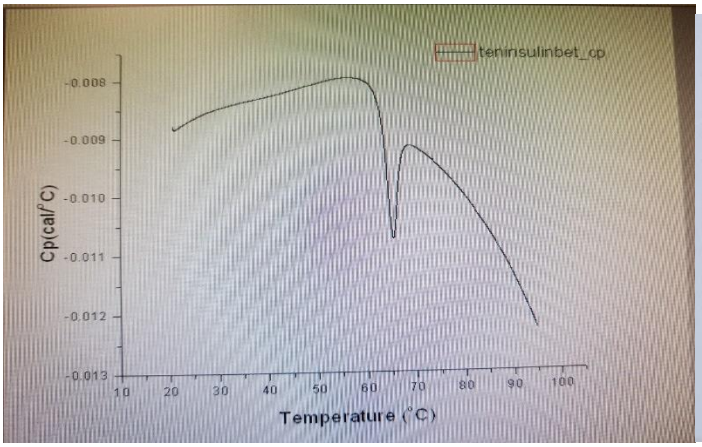

teninsuhcl050_cp
Cp(cal/°C)
-0.0020
-0.0025
-0.0030
-0.0035
-0.0040
-0.0045
10
20
30
40
50
60
70
80
90
100
Temperature (°C)

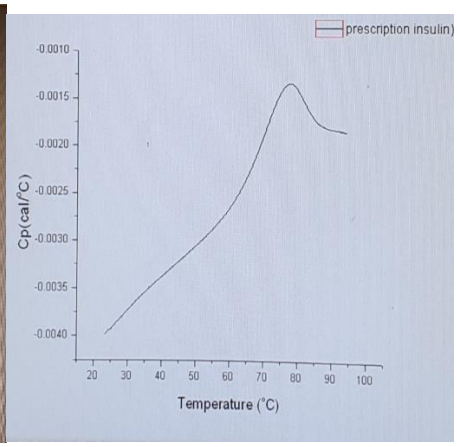

teninsulinbet_cp
-0.008
-0.009
-0.010
-0.011
-0.012
-0.013
Cp(cal/°C)
10
20
30
40
50
60
70
80
90
100
Temperature (°C)

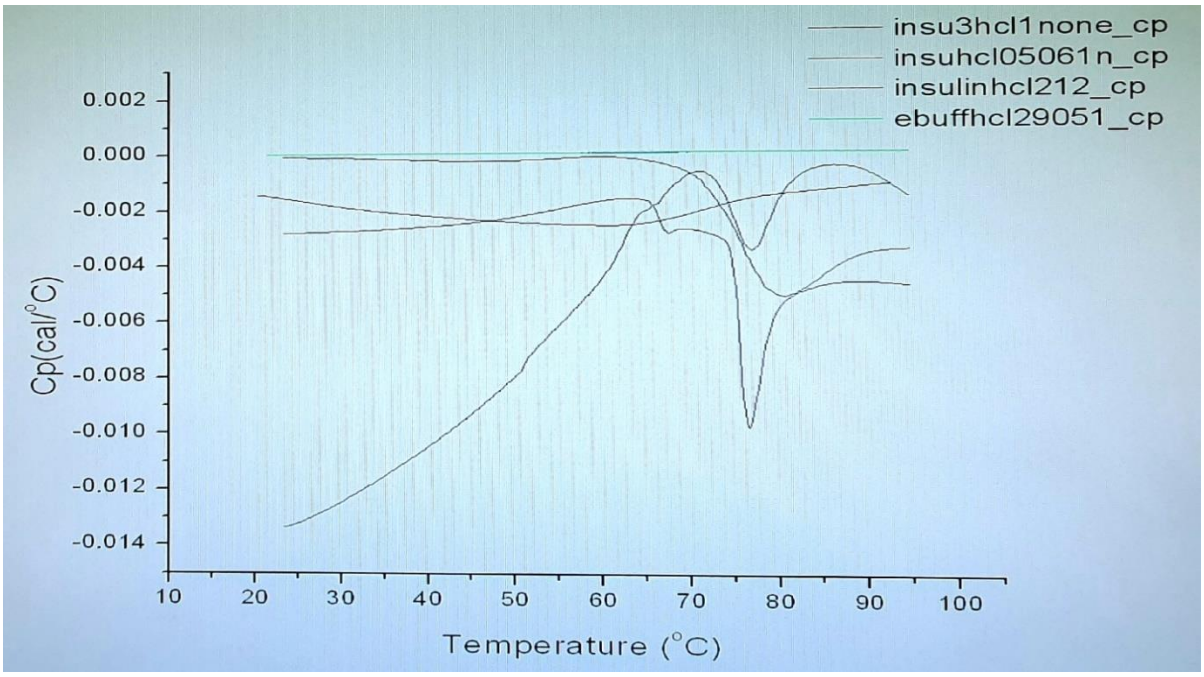

insuplur1306d_cp
-0.0015
-0.0020
-0.0025
-0.0030
-0.0035
-0.0040
-0.0045
-0.0050
Cp(cal/°C)
20
30
40
50
60
70
80
90
100
Temperature (°C)

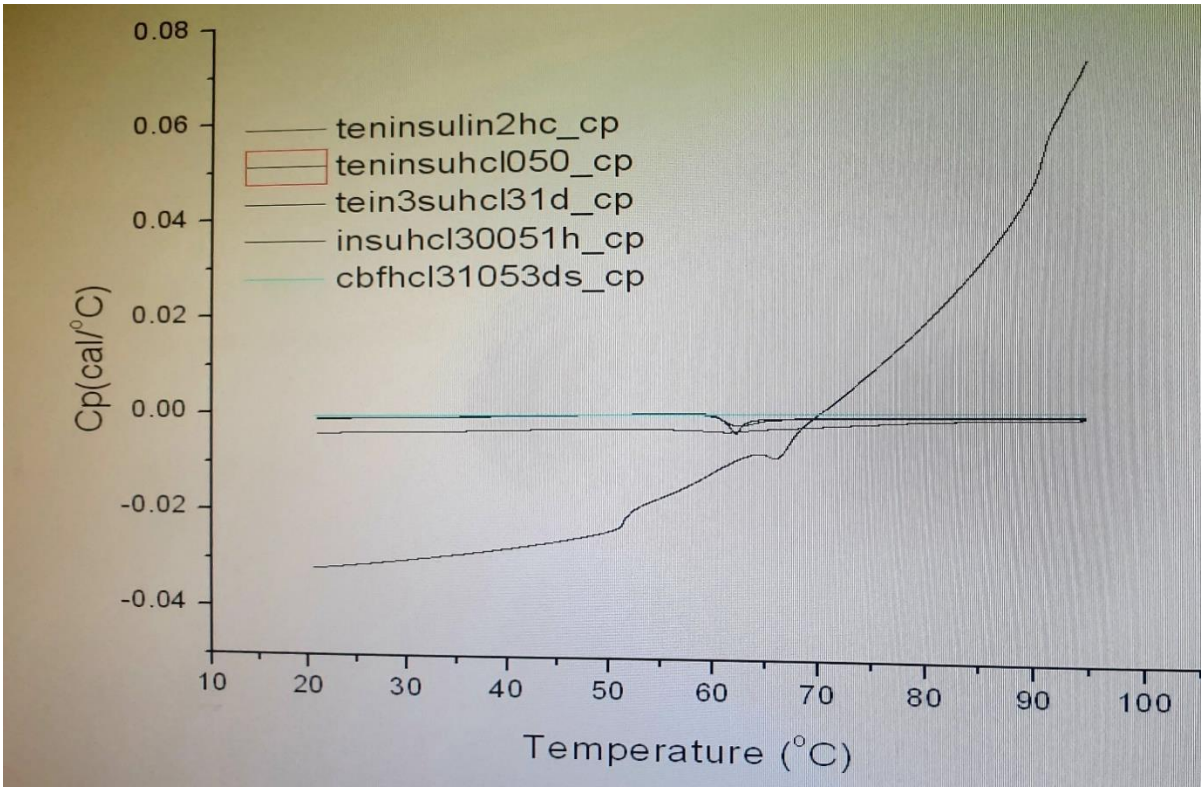

teninsulinplu_cp
Cp(cal/°C)
-0.002
-0.004
-0.006
-0.008
-0.010
-0.012
-0.014
10
20
30
40
50
60
70
80
90
100
Temperature (°C)

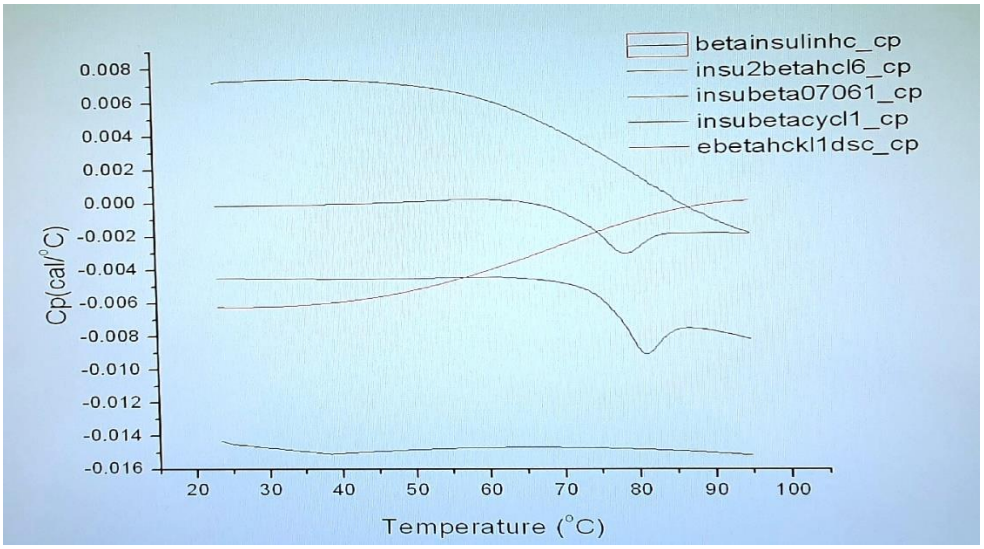

insubeta07061_cp
Cp(cal/°C)
-0.004
-0.005
-0.006
-0.007
-0.008
-0.009
-0.010
20
30
40
50
60
70
80
90
100
Temperature (°C)

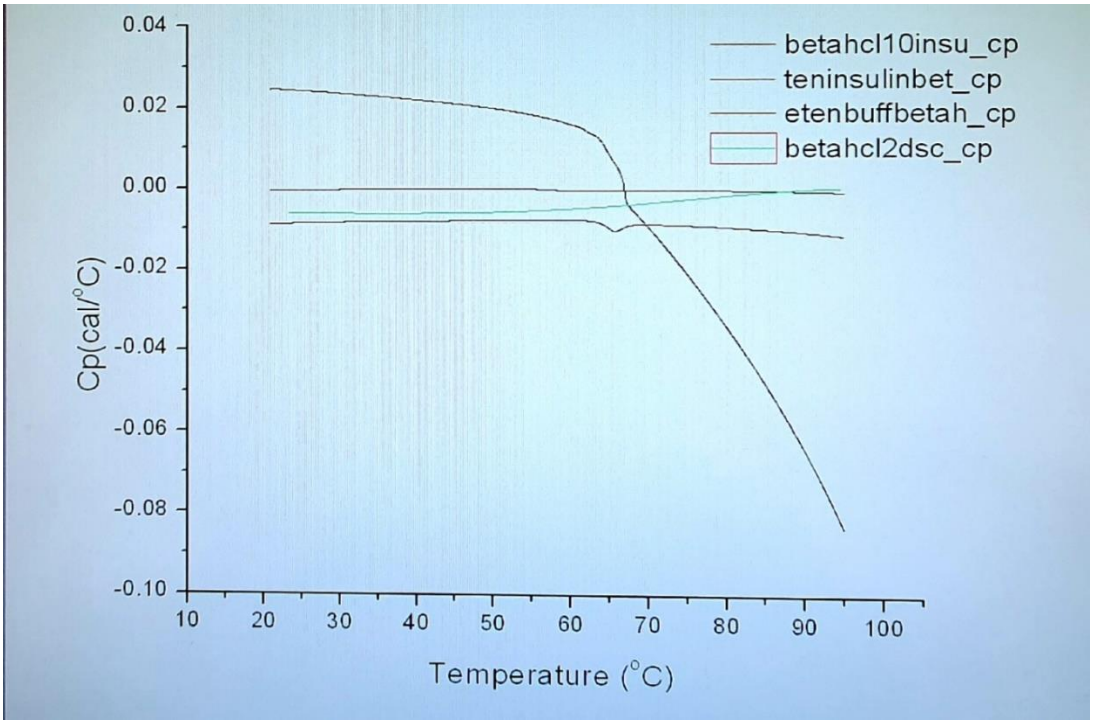

teninsulinbet_cp
Cp(cal/°C)
-0.008
-0.009
-0.010
-0.011
-0.012
-0.013
10
20
30
40
50
60
70
80
90
100
Temperature (°C)

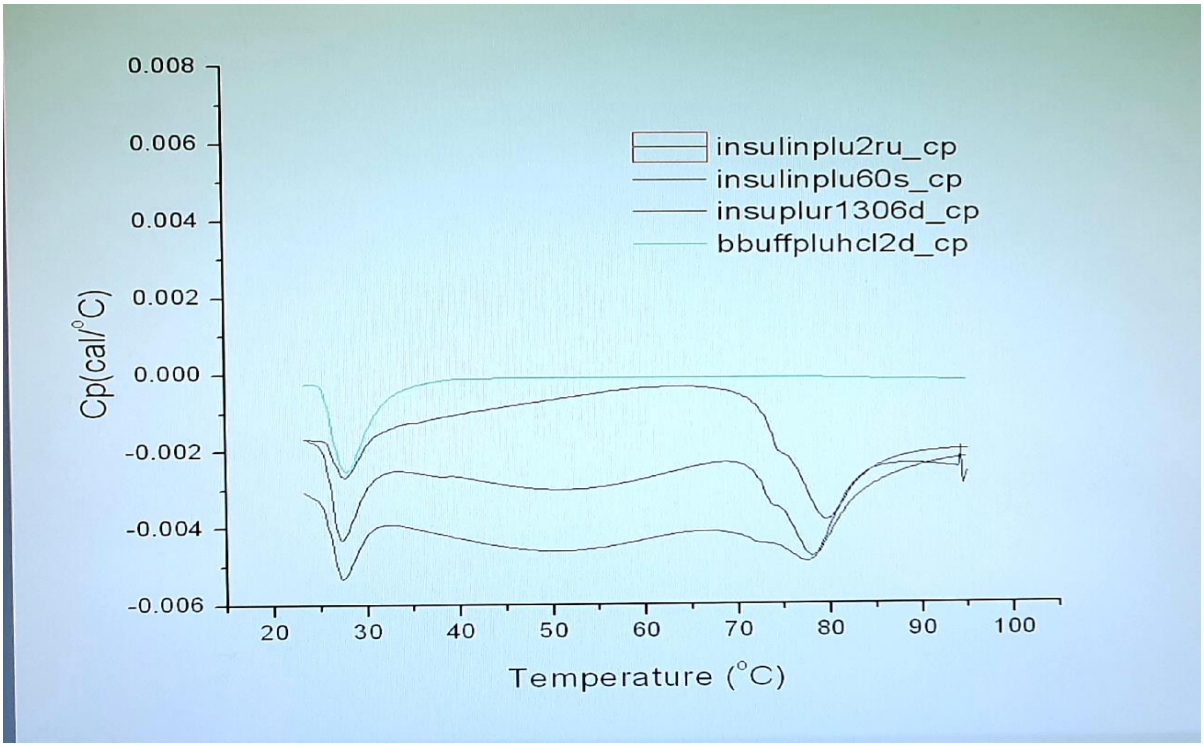

prescription insulin)
Cp(cal/°C)
-0.0010
-0.0015
-0.0020
-0.0025
-0.0030
-0.0035
-0.0040
20
30
40
50
60
70
80
90
100
Temperature (°C)

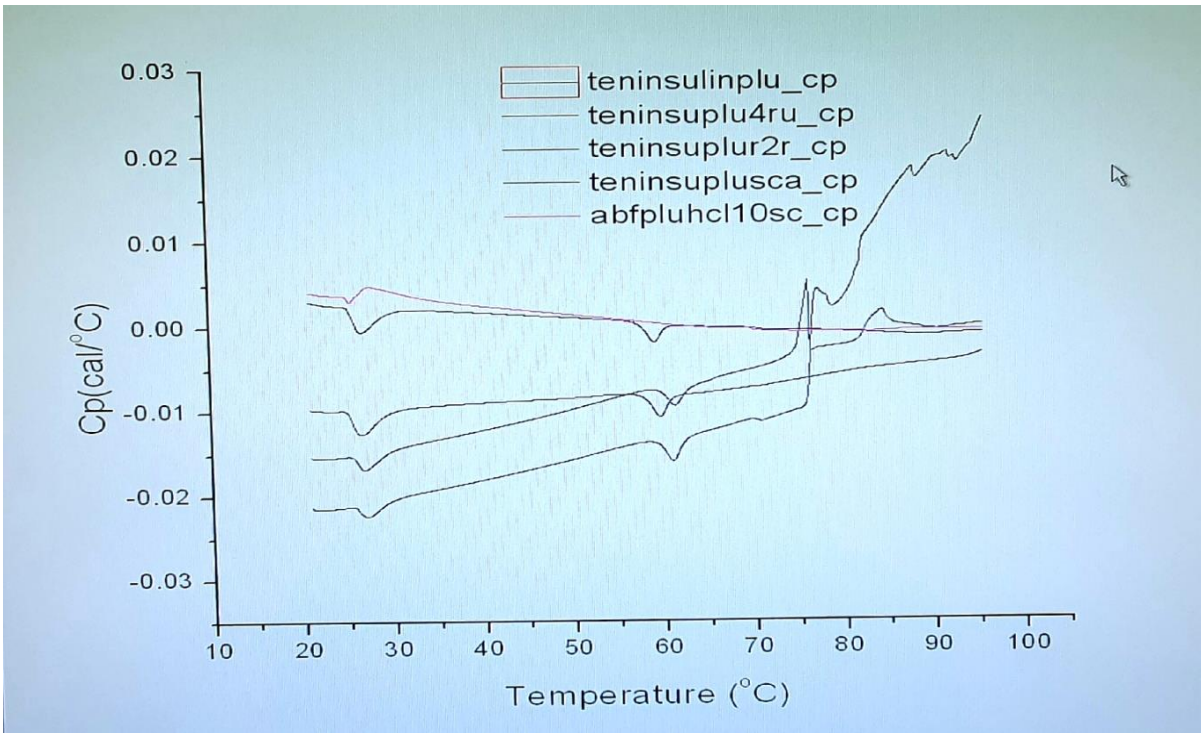

insu3hcl1none_cp
insuhcl05061n_cp
insulinhcl212_cp
ebuffhcl29051_cp
0.002
0.000
-0.002
-0.004
-0.006
-0.008
-0.010
-0.012
-0.014
Cp(cal/°C)
10
20
30
40
50
60
70
80
90
100
Temperature (°C)

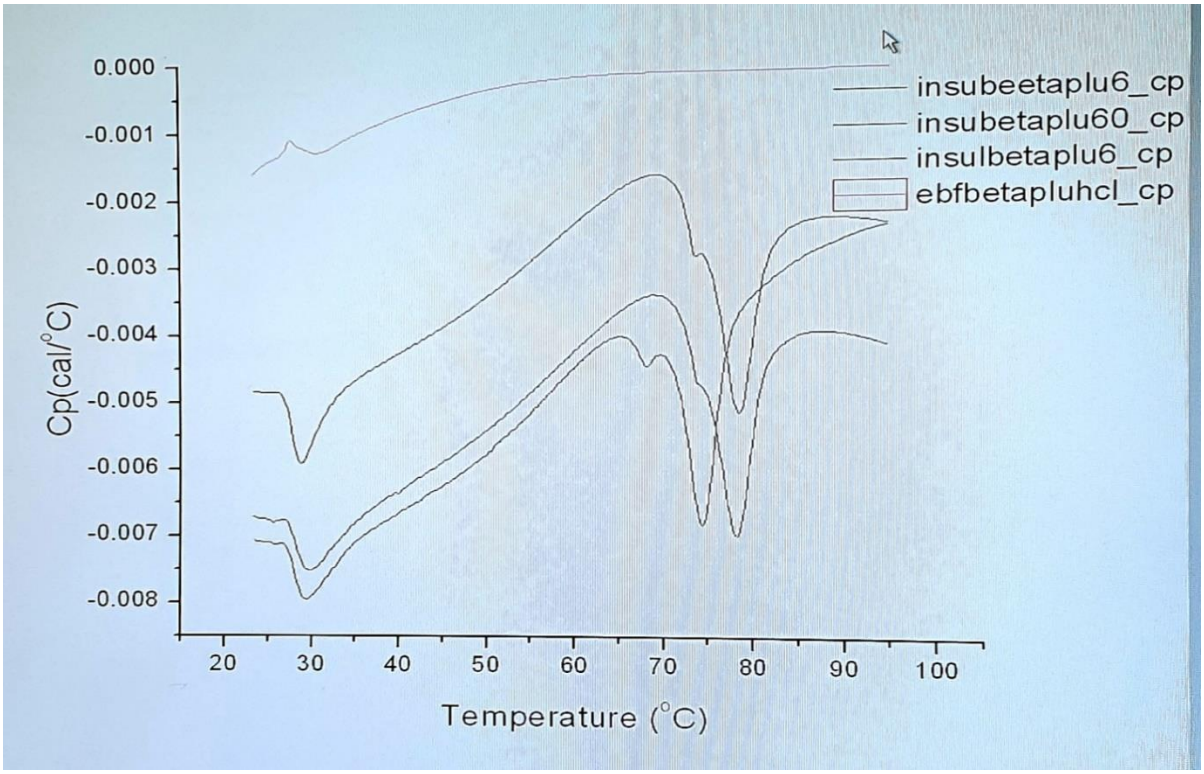

0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
Cp(cal/°C)
teninsulin2hc_cp
teninsuhcl050_cp
tein3suhcl31d_cp
insuhcl30051h_cp
cbfhcl31053ds_cp
10
20
30
40
50
60
70
80
90
100
Temperature (°C)

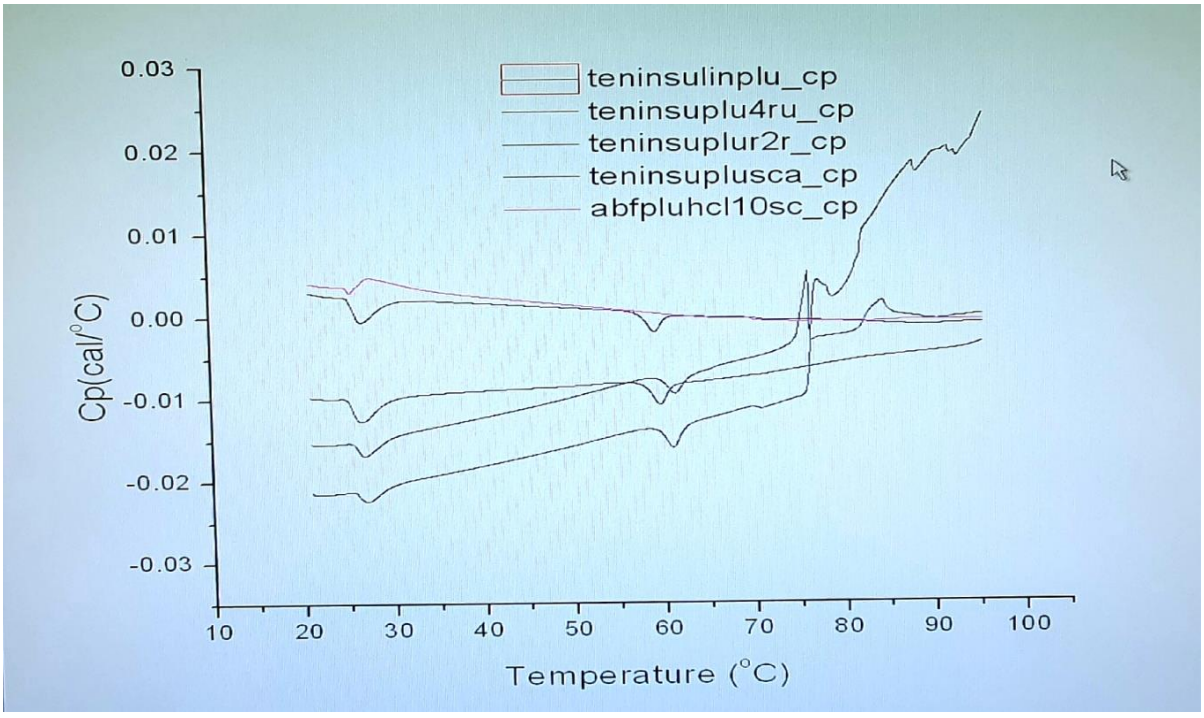

betainsulinhc_cp
insu2betahcl6_cp
insubeta07061_cp
insubetacycl1_cp
ebetahckl1dsc_cp
Cp(cal/°C)
0.008
0.006
0.004
0.002
0.000
-0.002
-0.004
-0.006
-0.008
-0.010
-0.012
-0.014
-0.016
20
30
40
50
60
70
80
90
100
Temperature (°C)

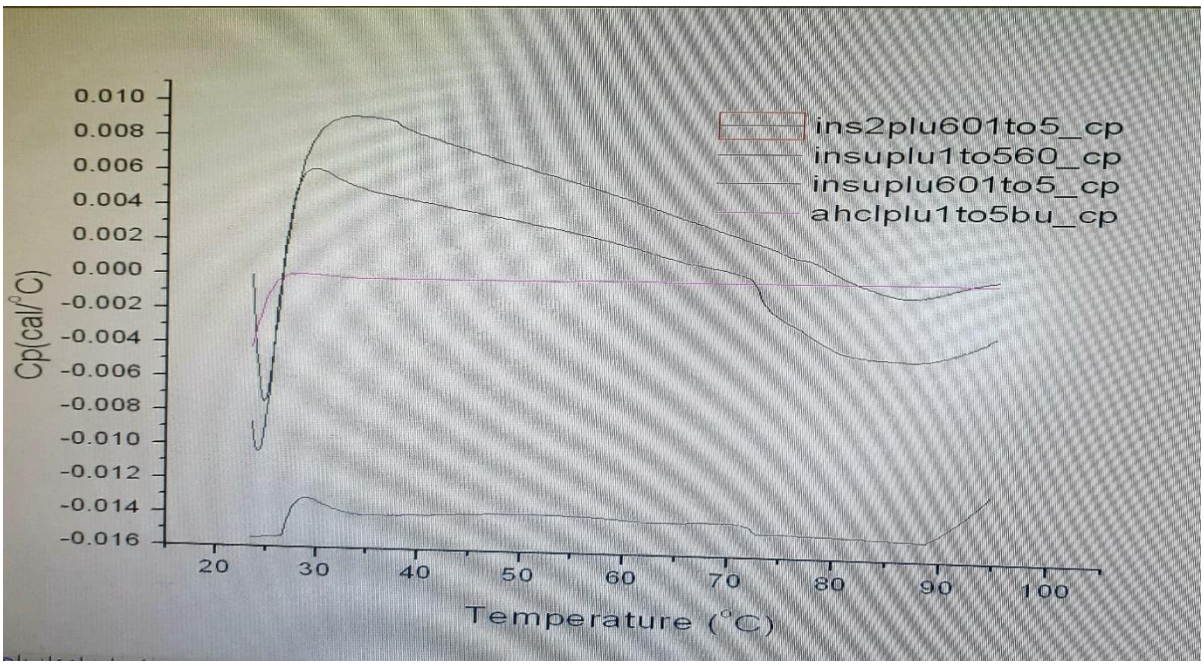

betahcl10insu_cp
teninsulinbet_cp
etenbuffbetah_cp
betahcl2dsc_cp
Cp(cal/°C)
0.04
0.02
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
10
20
30
40
50
60
70
80
90
100
Temperature (°C)

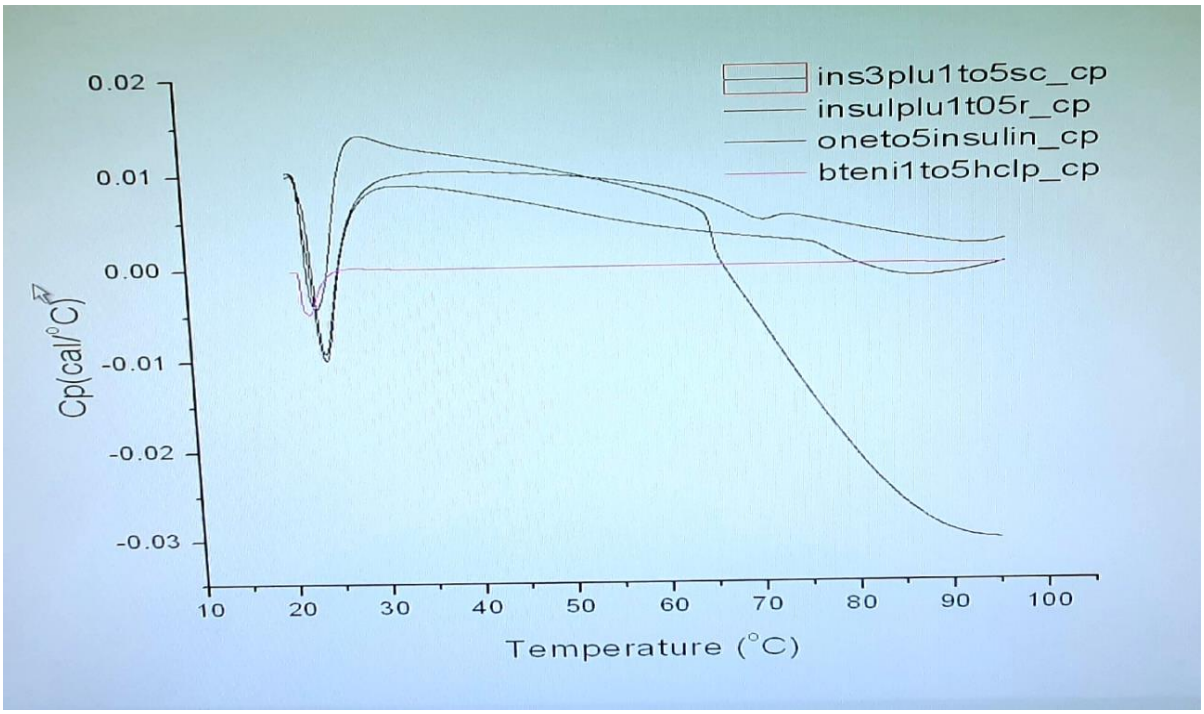

0.008
0.006
0.004
0.002
0.000
-0.002
-0.004
-0.006
Cp(cal/°C)
20
30
40
50
60
70
80
90
100
Temperature (°C)
insulinplu2ru_cp
insulinplu60s_cp
insuplur1306d_cp
bbuffpluhcl2d_cp

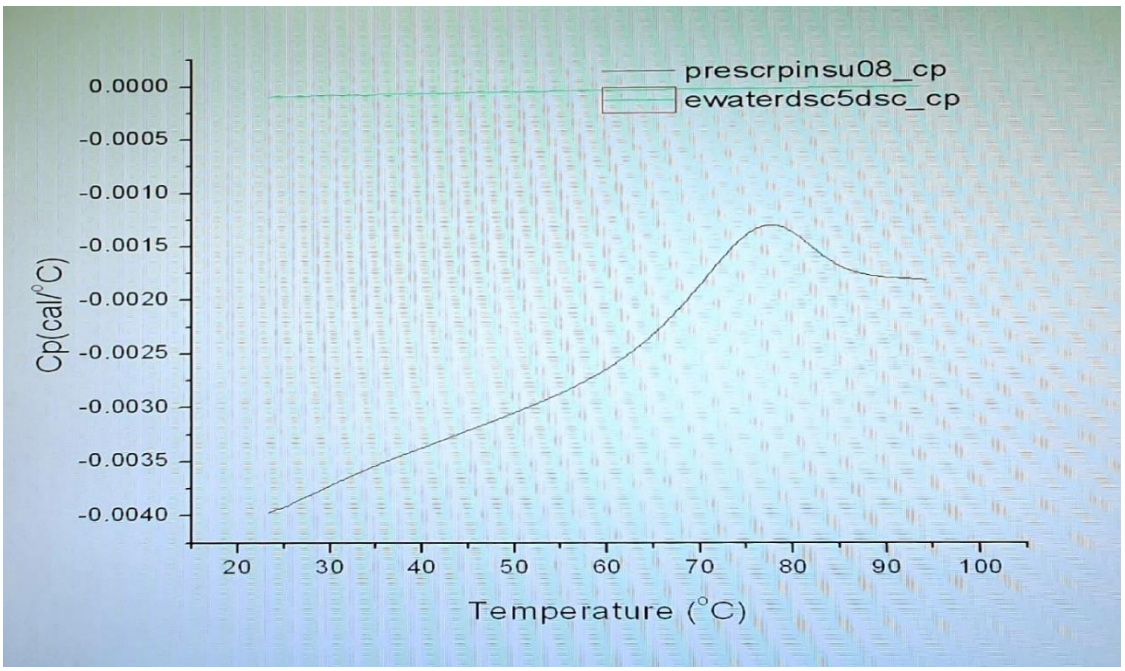

0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
Cp(cal/°C)
10
20
30
40
50
60
70
80
90
100
Temperature (°C)
teninsulinplu_cp
teninsuplu4ru_cp
teninsuplur2r_cp
teninsuplusca_cp
abfpluhcl10sc_cp

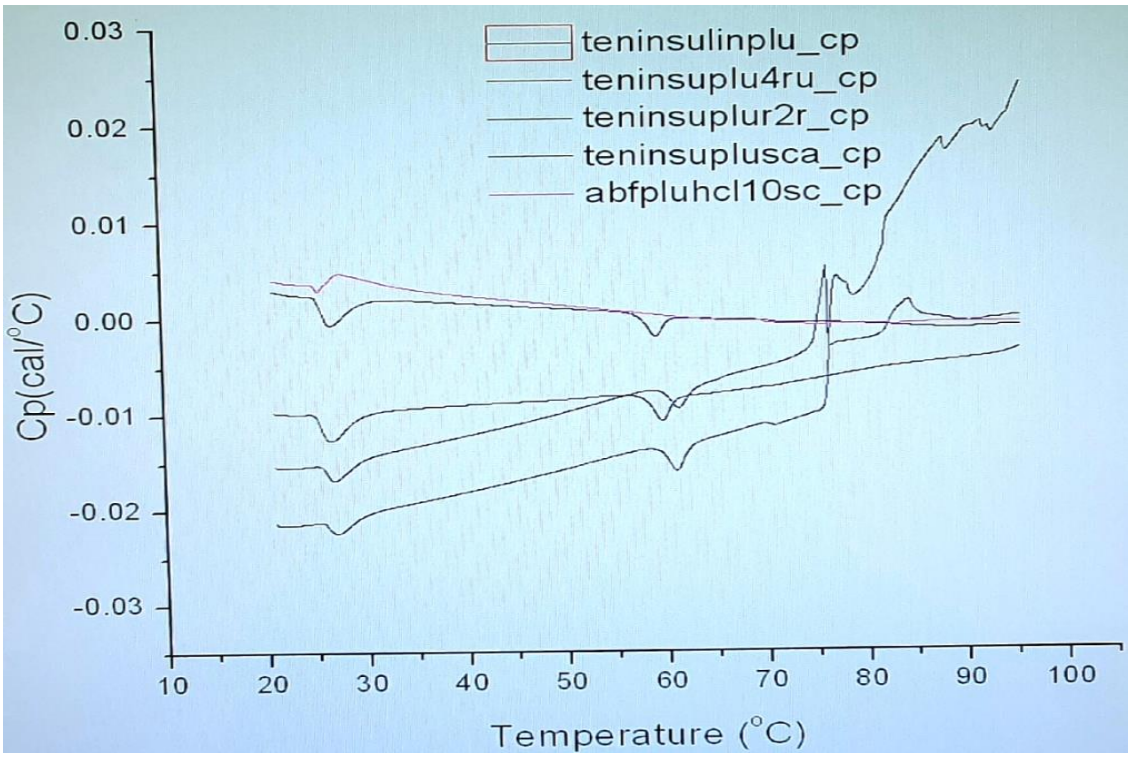

insubeetaplu6_cp
insubetaplu60_cp
insulbetaplu6_cp
ebfbetapluhcl_cp
Cp(cal/°C)
0.000
-0.001
-0.002
-0.003
-0.004
-0.005
-0.006
-0.007
-0.008
20
30
40
50
60
70
80
90
100
Temperature (°C)


teninsulinplu_cp
teninsuplu4ru_cp
teninsuplur2r_cp
teninsuplusca_cp
abfpluhcl10sc_cp
Cp(cal/°C)
0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
10
20
30
40
50
60
70
80
90
100
Temperature (°C)

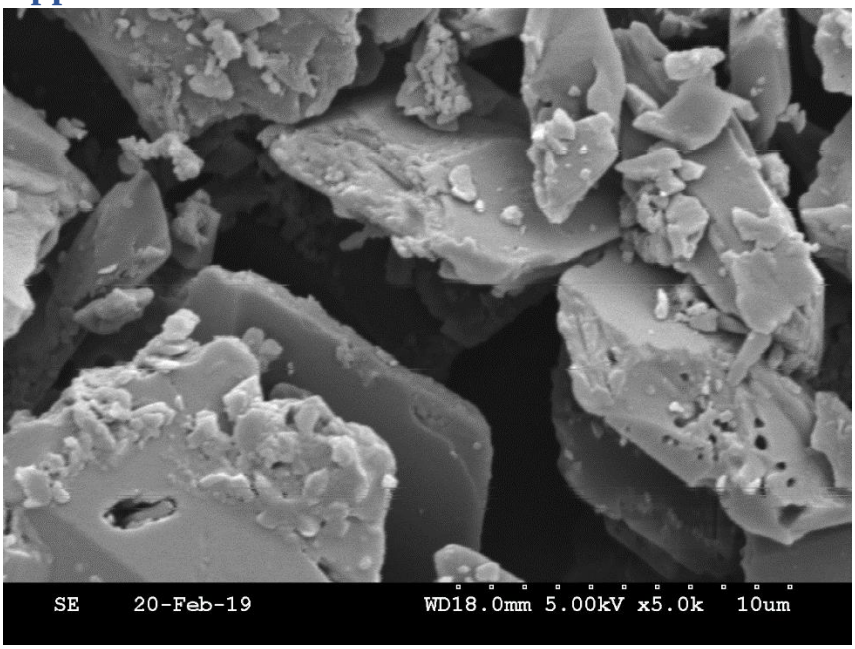

ins2plu601to5_cp
insuplu1to560_cp
insuplu601to5_cp
ahclplu1to5bu_cp
Cp(cal/°C)
0.010
0.008
0.006
0.004
0.002
0.000
-0.002
-0.004
-0.006
-0.008
-0.010
-0.012
-0.014
-0.016
20
30
40
50
60
70
80
90
100
Temperature (°C)


ins3plu1to5sc_cp
insulplu1t05r_cp
oneto5insulin_cp
bteni1to5hclp_cp
Cp(cal/°C)
0.02
0.01
0.00
-0.01
-0.02
-0.03
10
20
30
40
50
60
70
80
90
100
Temperature (°C)

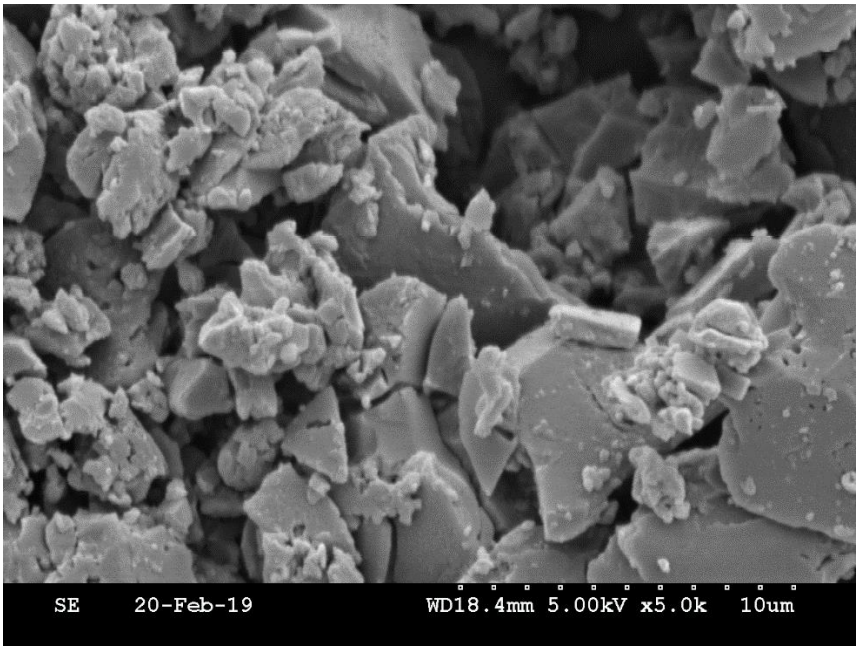

prescrpinsu08_cp
ewaterdsc5dsc_cp
0.0000
-0.0005
-0.0010
-0.0015
-0.0020
-0.0025
-0.0030
-0.0035
-0.0040
Cp(cal/°C)
20
30
40
50
60
70
80
90
100
Temperature (°C)


teninsulinplu_cp
teninsuplu4ru_cp
teninsuplur2r_cp
teninsuplusca_cp
abfpluhcl10sc_cp
0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
Cp(cal/°C)
10
20
30
40
50
60
70
80
90
100
Temperature (°C)

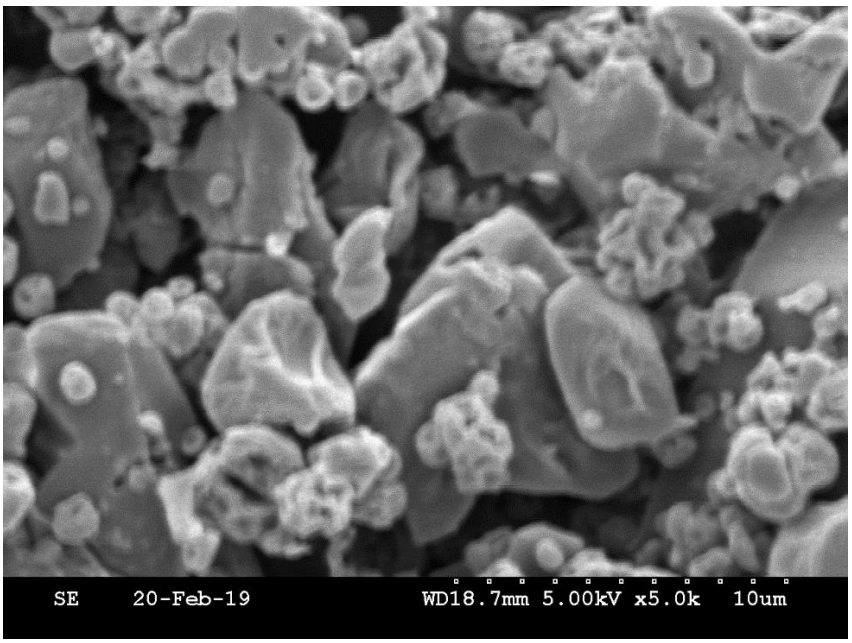
Appendices x: SEM data for insulin



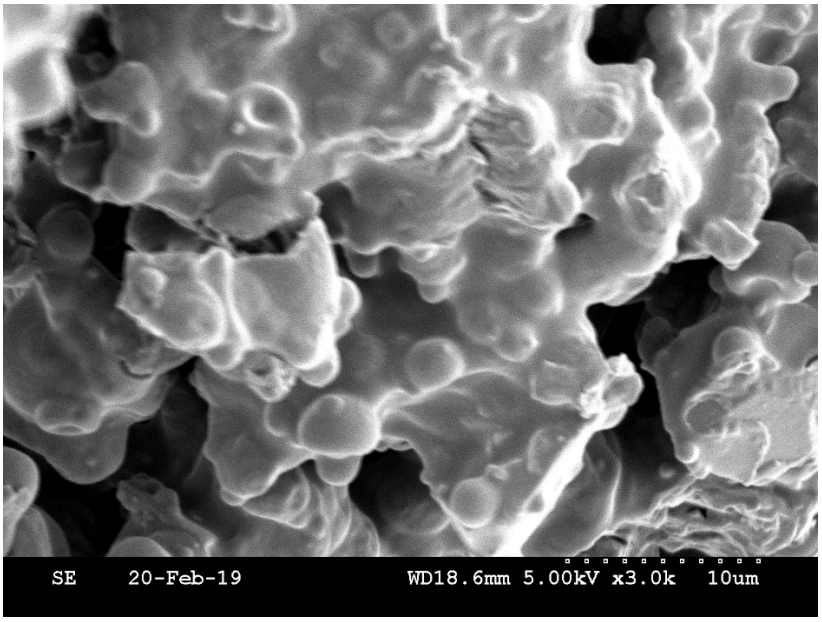
Pure insulin



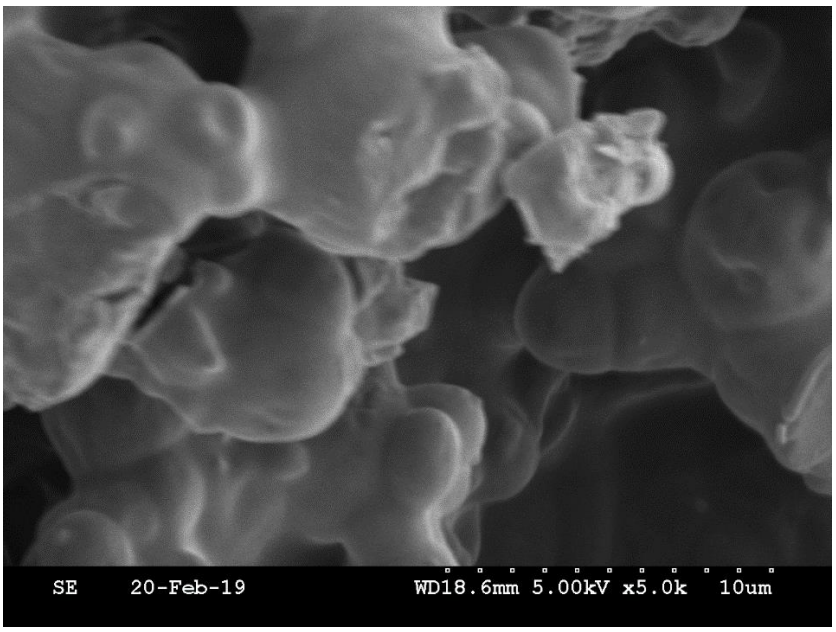
spraydried insulin



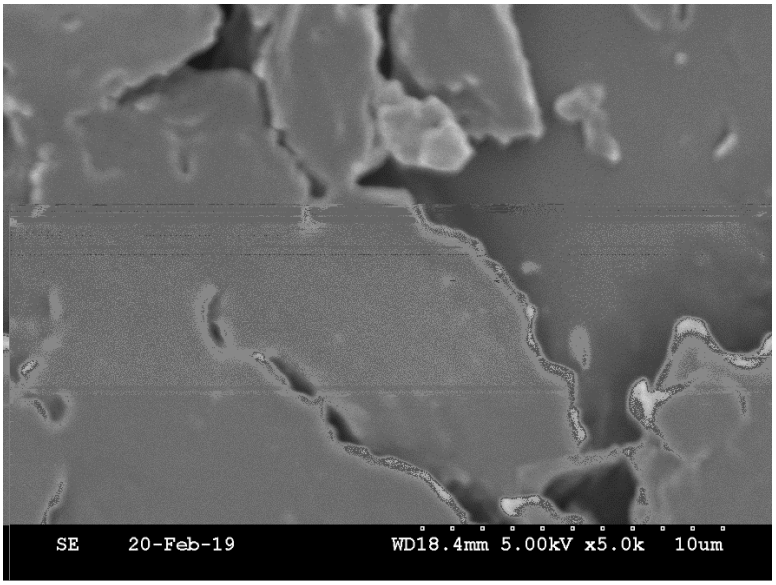
Spraydry insulin betacyclodextrin



insulin betacyclodextrin pluronic



insulin betacyclodextrin pluronic



insulin with pluronic

Appendix xi: Thermogram to Monoclonal antibody

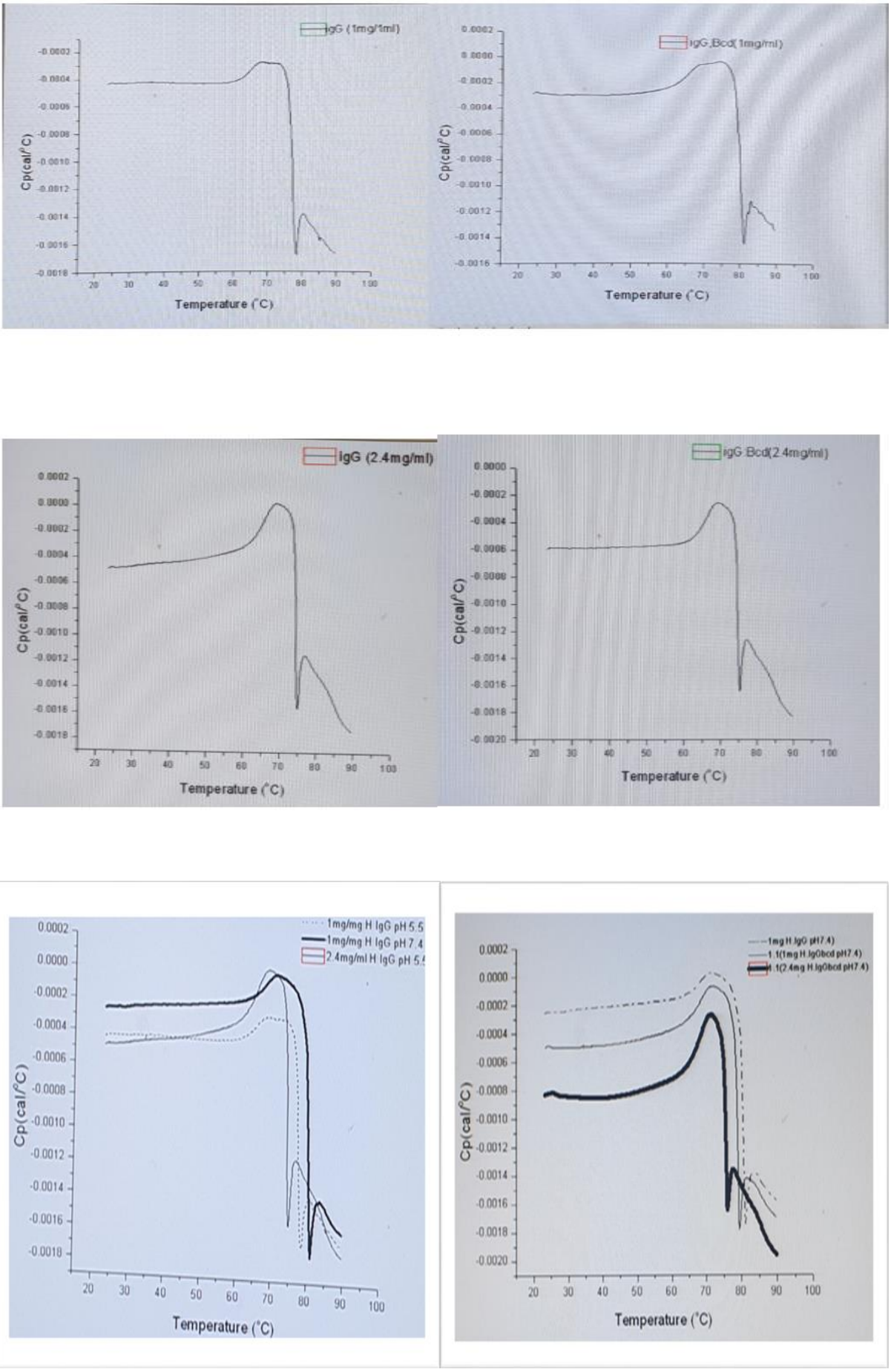
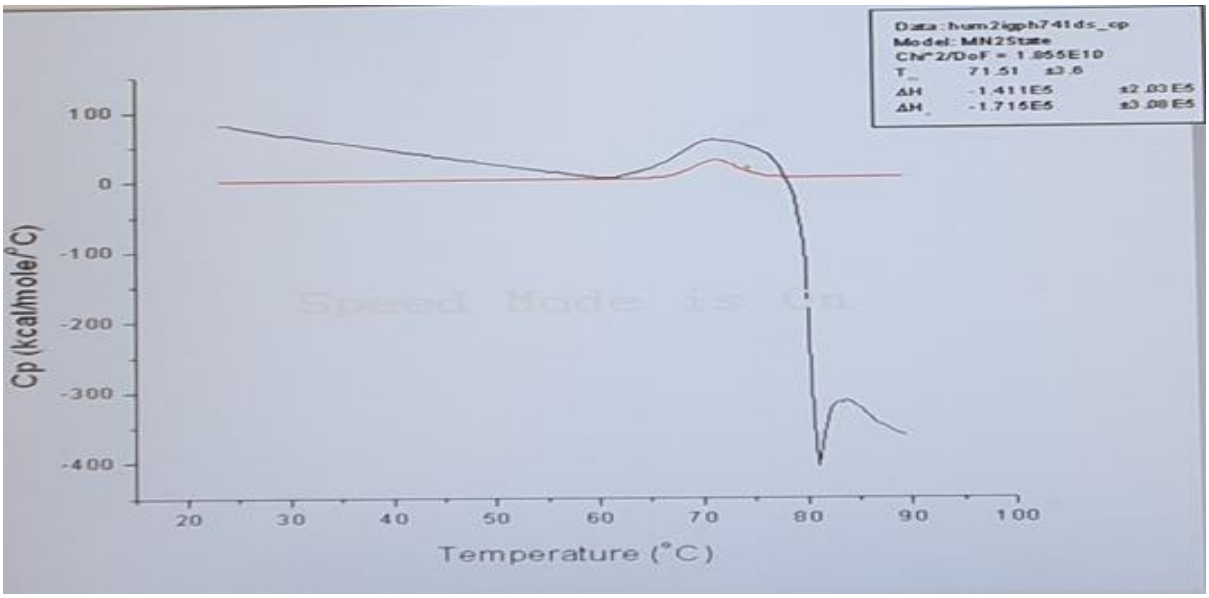
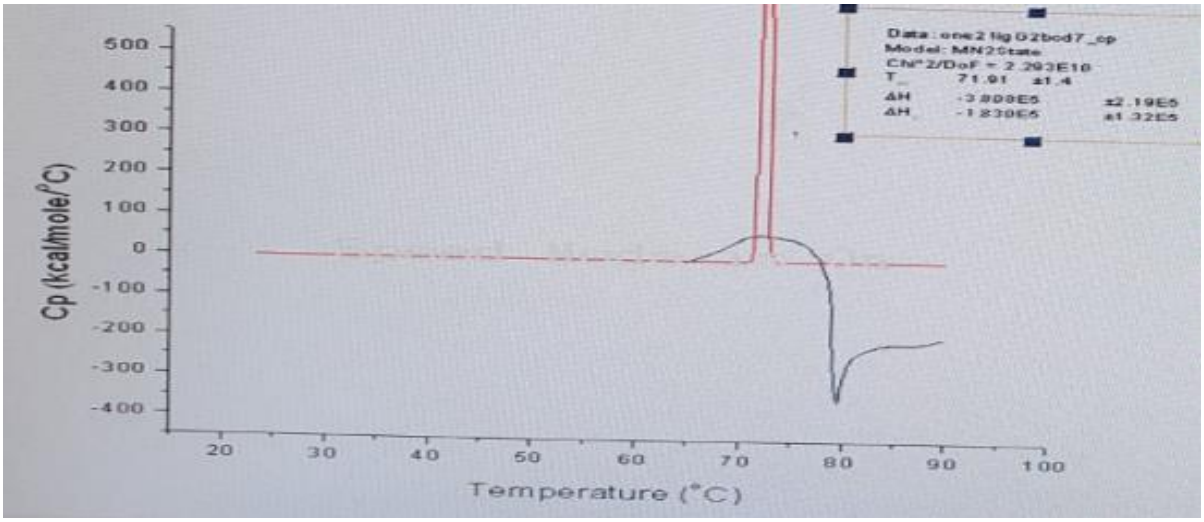


Figure 5.3 Raw DSC thermogram of human IgG (1 mg/ml;2mg/ml) in a 10-mM sodium citrate, 140mM Nacl buffer, pH 5.5 and 7.4



non two state thermogram of human IgG (1 mg/ml) in a 10-mM sodium citrate, 140mM Nacl buffer, pH 7.4



Appendix xiii **Statistical Analysis**

One way

Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	Minimum	Maximum

						Lower Bound	Upper Bound		
eight month	noexcipient	3	24.4933	42.42370	24.49333	-80.8930	129.8796	.00	73.48
	betacyclodextrin	3	24.5367	42.49875	24.53667	-81.0361	130.1094	.00	73.61
	pluronic	3	24.7067	42.79320	24.70667	-81.5975	131.0109	.00	74.12
	betacyclodextrin+pluronic	3	24.8433	43.02992	24.84333	-82.0489	131.7356	.00	74.53
	Total	12	24.6450	36.40395	10.50892	1.5150	47.7750	.00	74.53
zero month	noexcipient	3	48.9267	42.38194	24.46923	-56.3559	154.2092	.00	74.32
	betacyclodextrin	3	49.2267	42.63472	24.61516	-56.6838	155.1372	.00	74.36
	pluronic	3	49.5167	42.88603	24.76026	-57.0181	156.0515	.00	74.81
	betacyclodextrin+pluronic	3	49.1400	42.55967	24.57183	-56.5841	154.8641	.00	74.23
	Total	12	49.2025	36.34370	10.49152	26.1108	72.2942	.00	74.81

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
eight month	Between Groups	.234	3	.078	.000	1.000
	Within Groups	14577.492	8	1822.186		
	Total	14577.726	11			
zero month	Between Groups	.538	3	.179	.000	1.000
	Within Groups	14528.969	8	1816.121		
	Total	14529.507	11			

One way

Descriptives

zero month

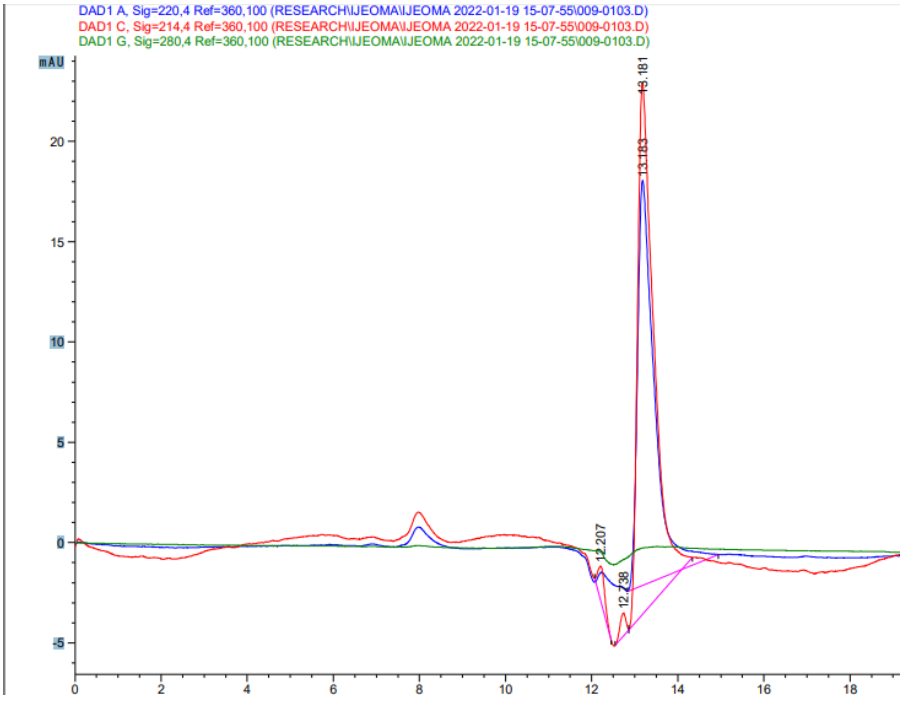
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
nodrying	4	73.6425	.50901	.25451	72.8326	74.4524	73.19	74.32
spraydrying	8	36.9825	39.54175	13.98012	3.9248	70.0402	.00	74.81
Total	12	49.2025	36.34370	10.49152	26.1108	72.2942	.00	74.81

ANOVA

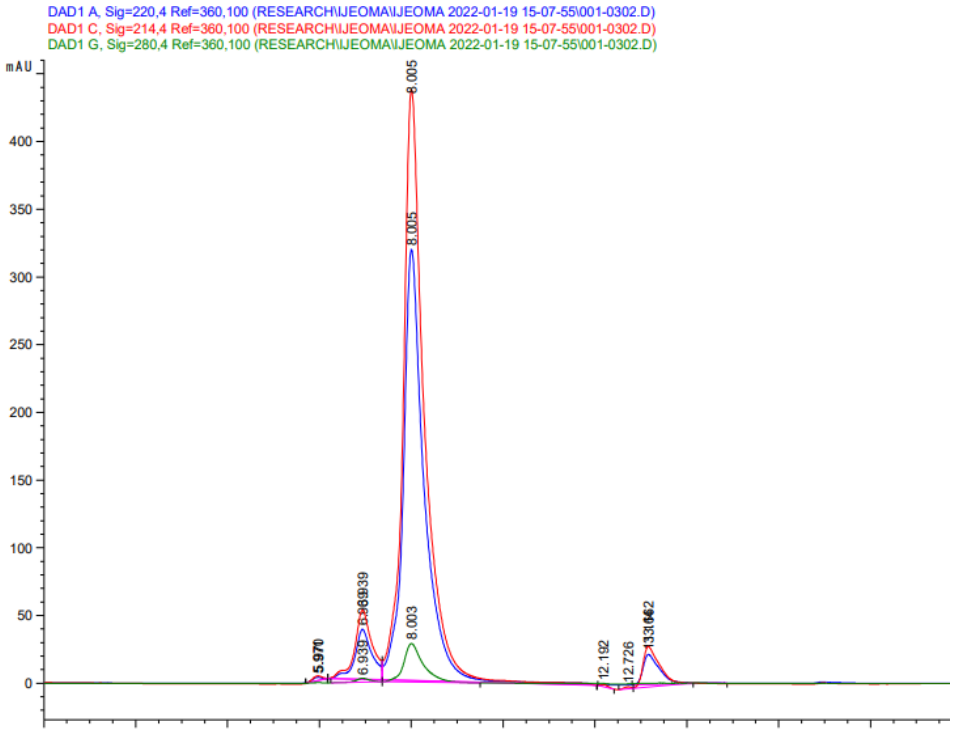
zero month

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3583.882	1	3583.882	3.274	.100
Within Groups	10945.625	10	1094.563		
Total	14529.507	11			

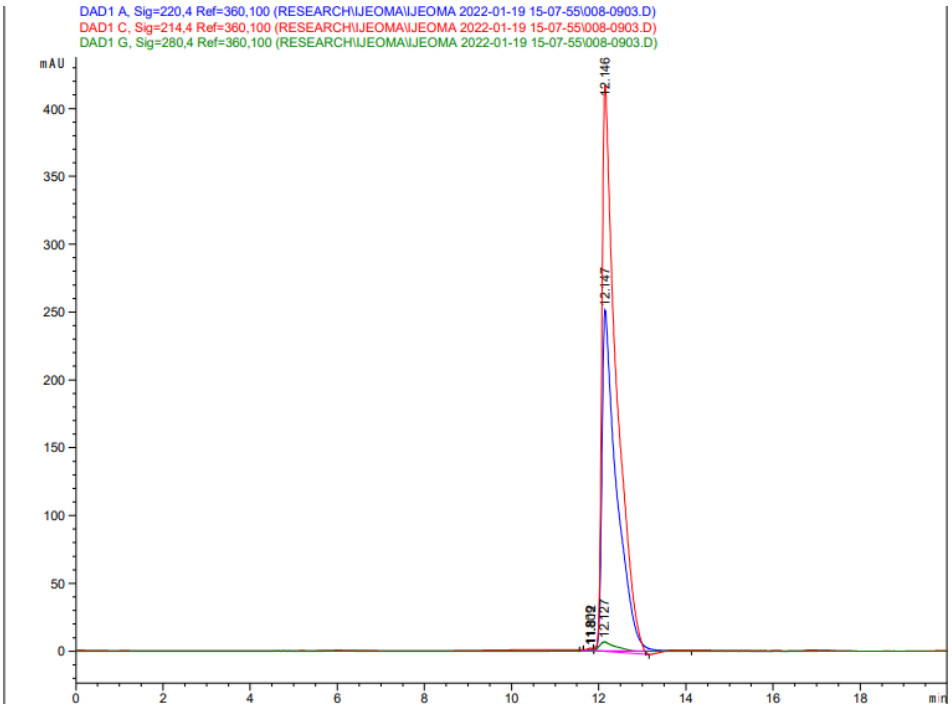
Appendix xii: **Chromatograms for Hplc data**



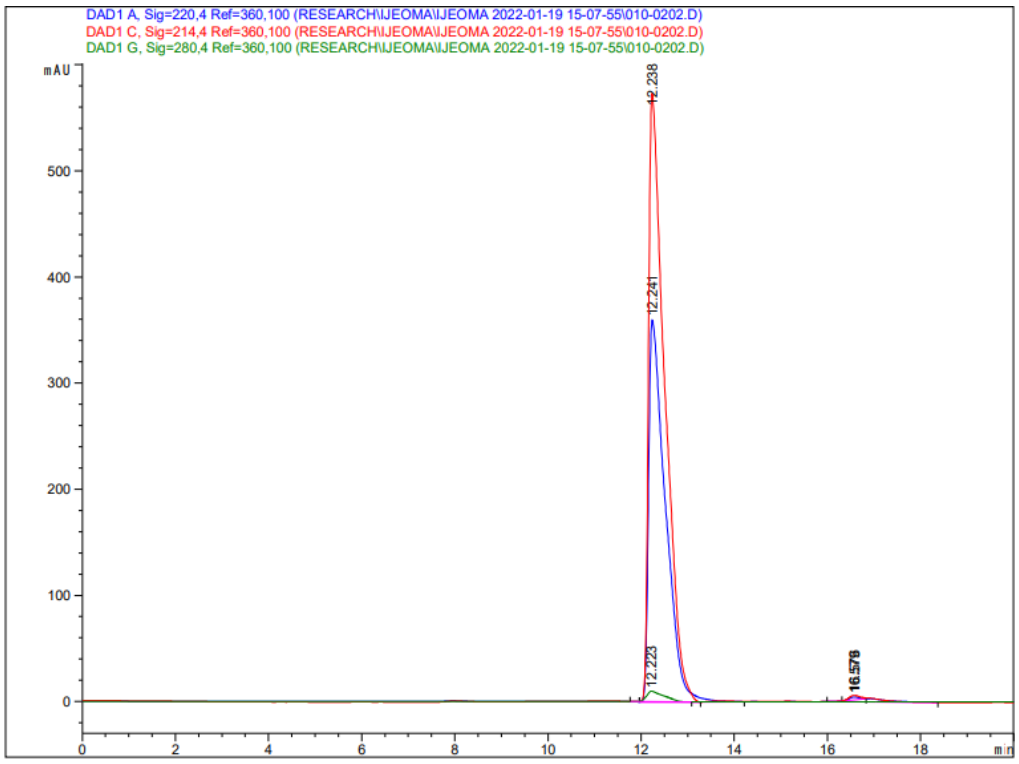
Water



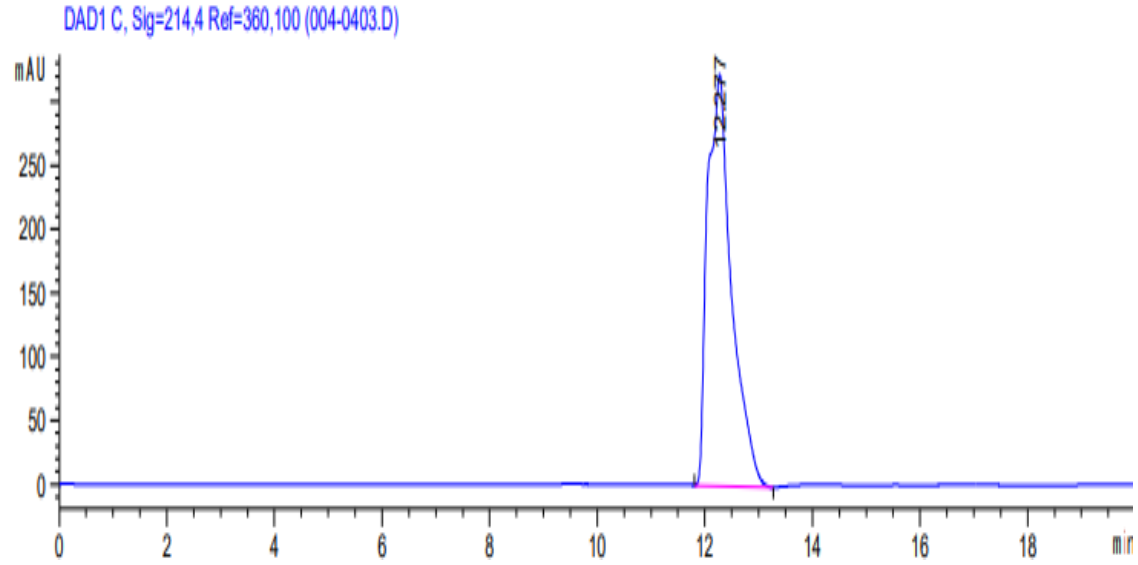
IgG in water



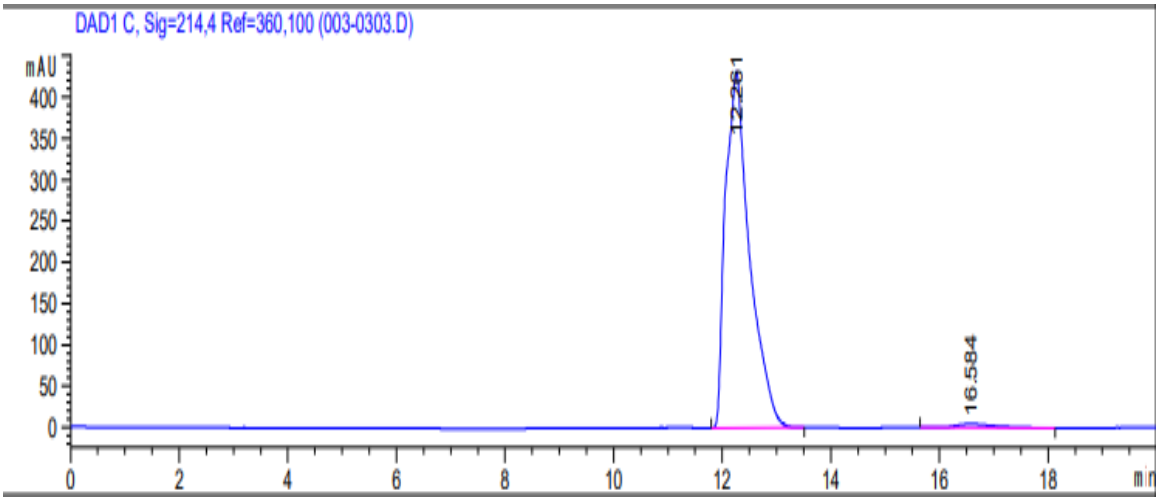
Sodium citrate buffer pH 7.4



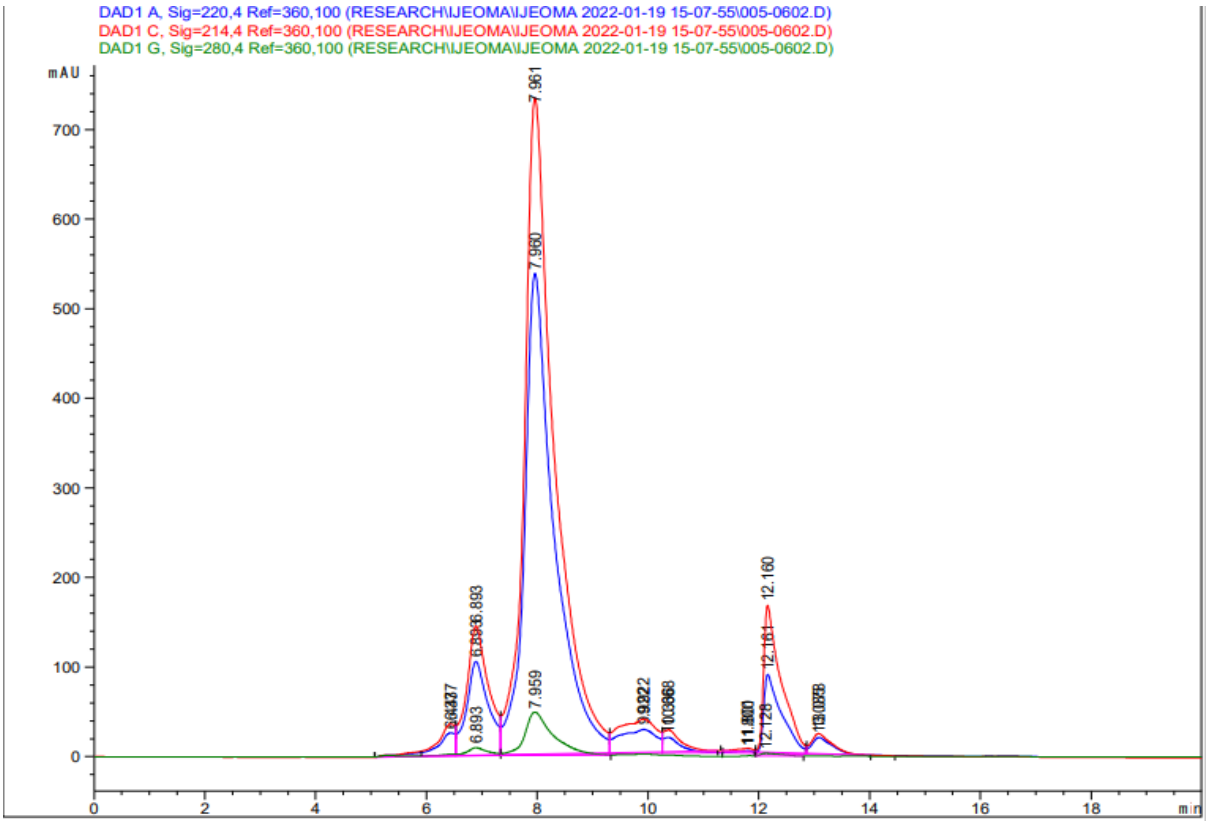
Sodium citrate buffer pH 5.5



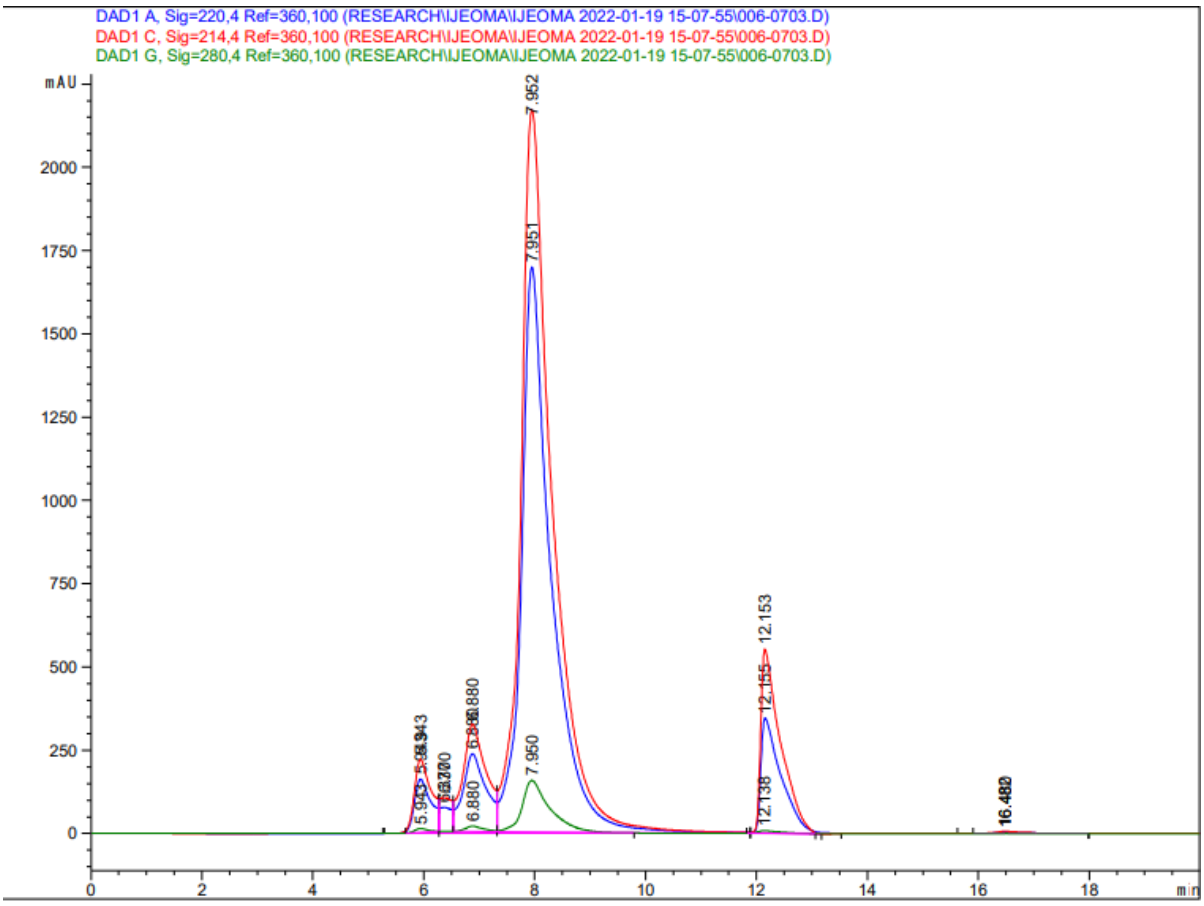
BCD buffer 7.4

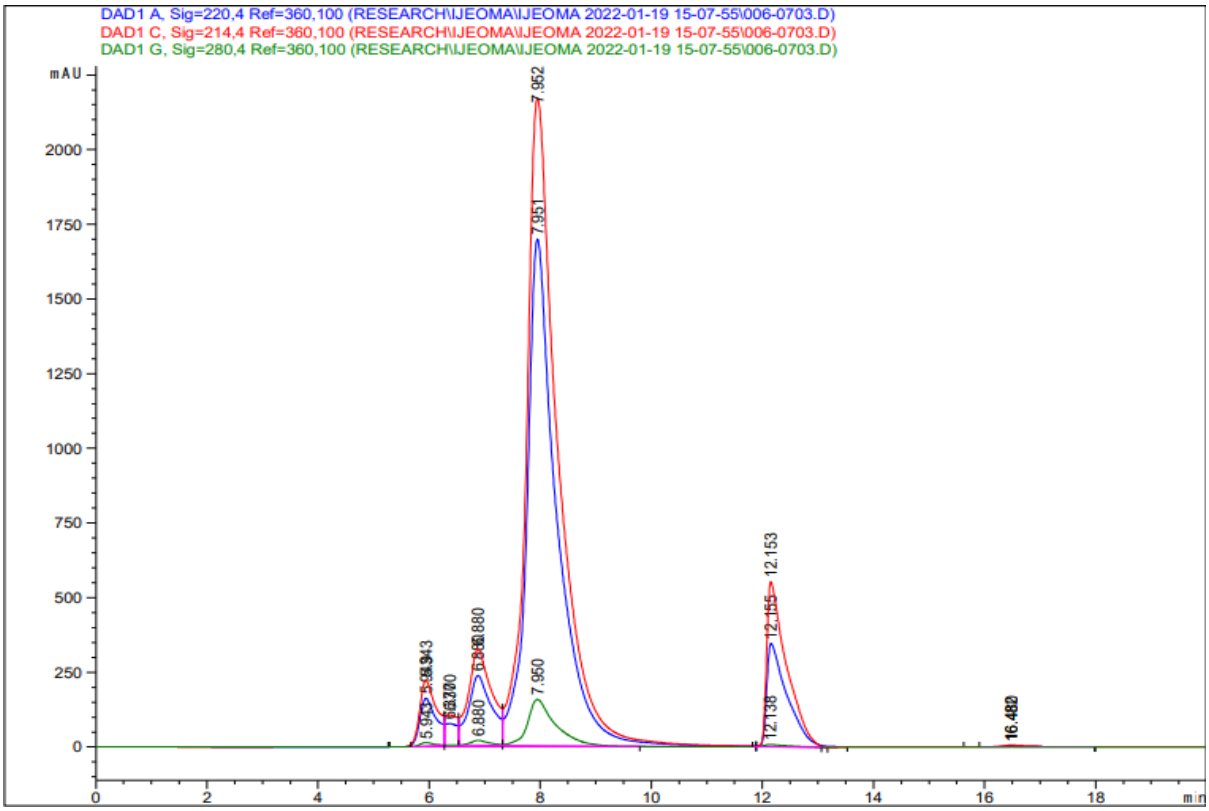


BCD buffer 5.5

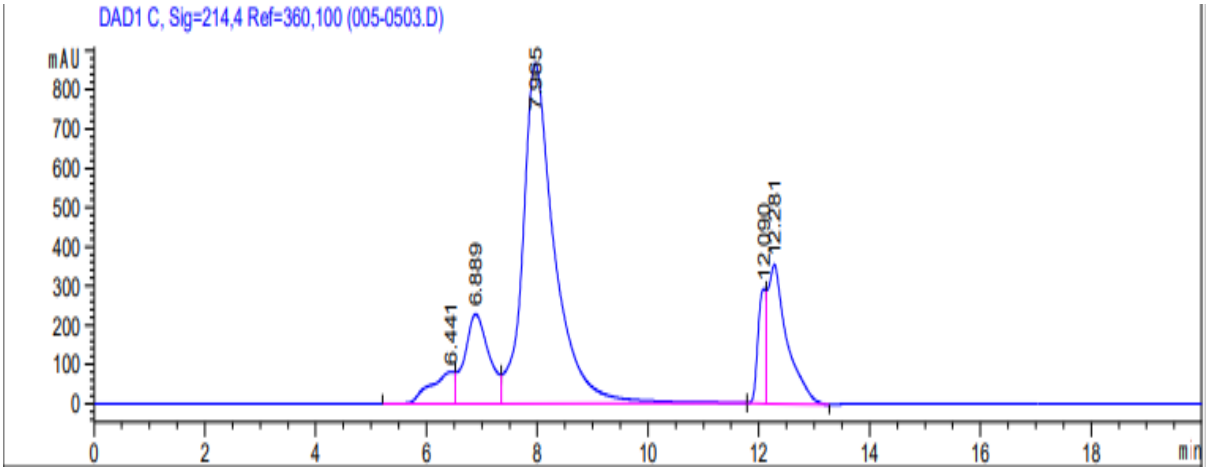


HEATED IGG 7.4

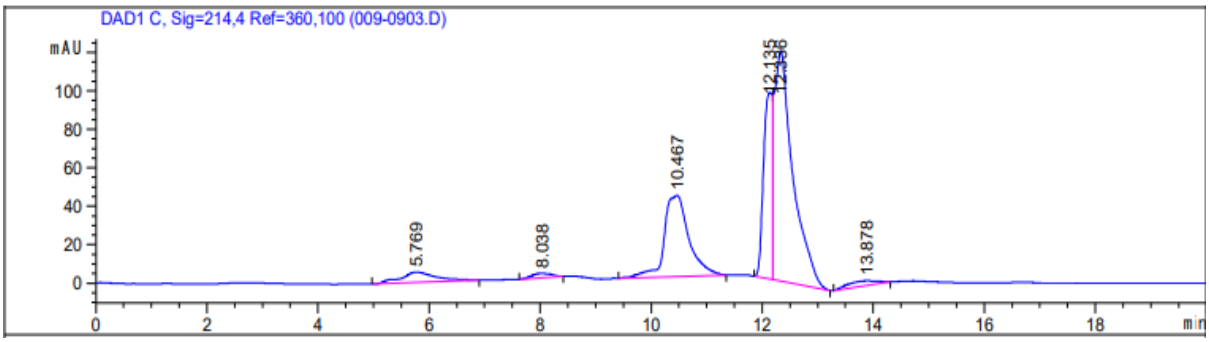




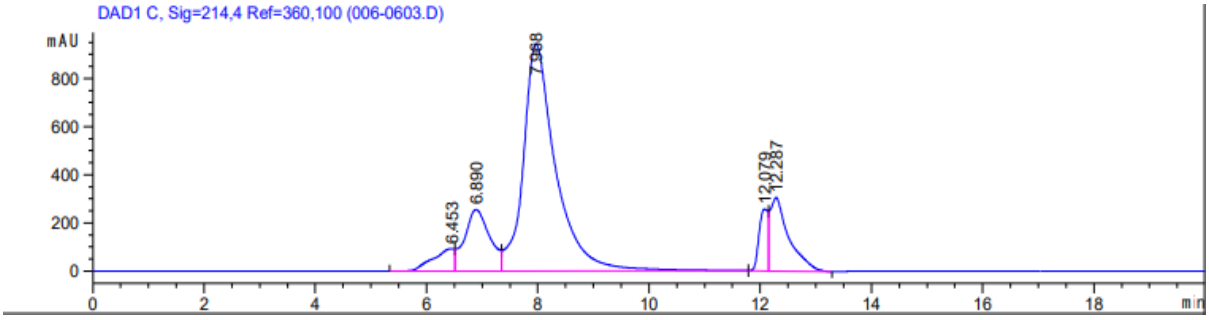
2.4mg citrate buffer 5.5



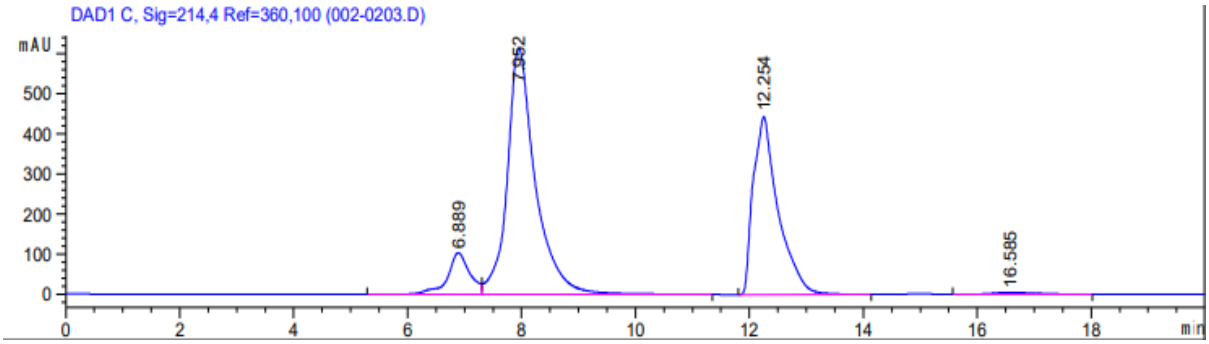
1:1 IgG beta (1mg) 7.4



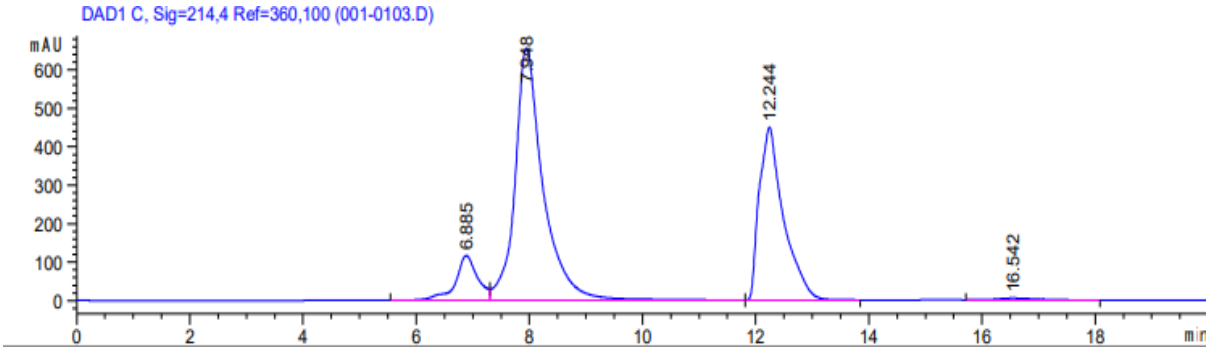
heated 1:1 (1mg)IgGbeta 7.4



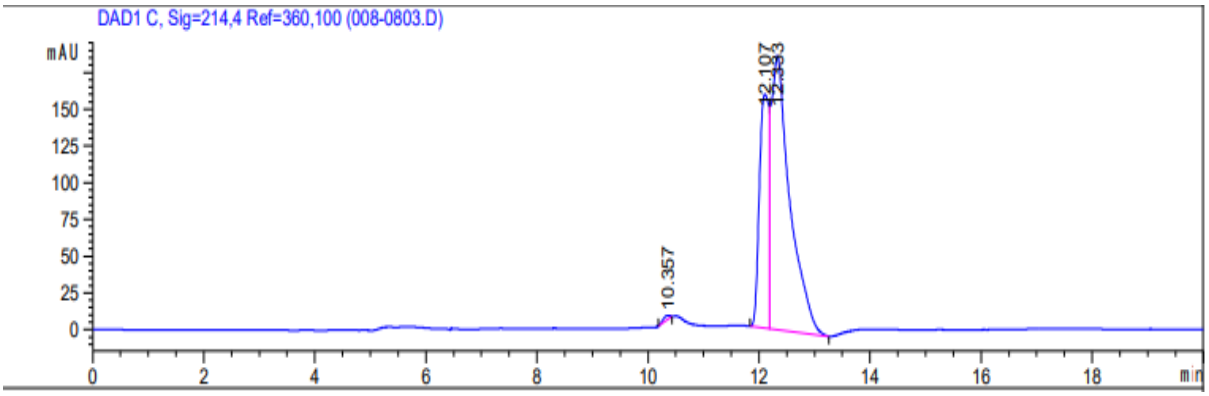
1:1 IgG beta (2.4mg) 7.4



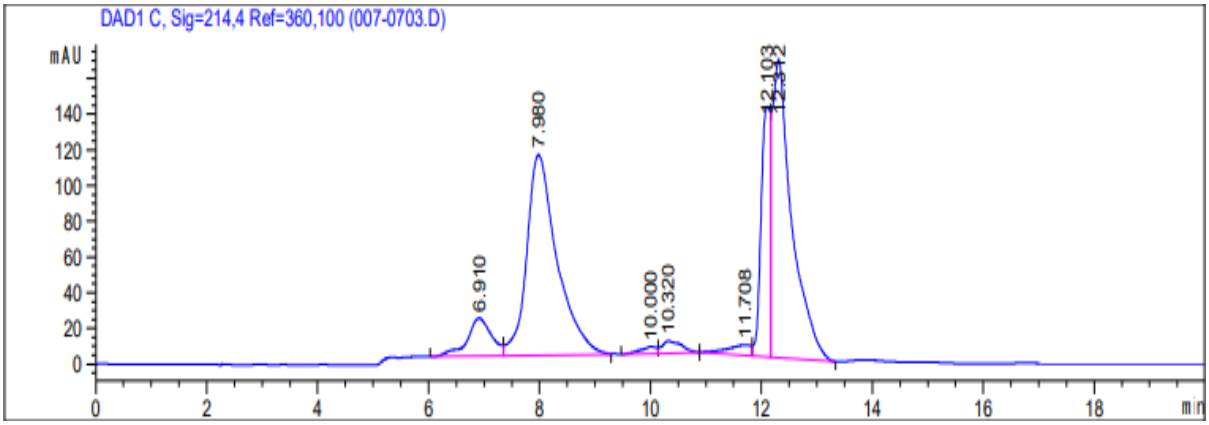
1:1 IgG beta (2.4mg) 5.5



1:1 IgG beta (1mg) 5.5



HEATED1mg IgG 7.4



HEAT 1:1 IgG beta 2.4mg 7.4

Appendix xiv: Personal Development

University of Sunderland

- Induction 26th May 2016
- Reporting on your research and developing your writing skills
- Research Fridays
- Kickstart your dissertation 10th June 2016

- Managing references using zotero 10th June, 2016
- COSHH and Bio-COSHH training
- Writing your thesis
- Faculty seminars
- Support for Doing a literature review-20th june, 2016
- The Cross Disciplinary Academic Writing programme- 15th June 2016
- Reporting on your research-13th June
- Academic Writing -5th,22nd ,29th of June and 6th July 2016
- Theoretical underpinnings, Research Methods, Health and Safety, 12th July, 2016
- Microsoft office for creating researching reports and your thesis May 18. 2019
- Completing your Research Degree May 13 & 14 2019. May 2 2020

- **TRAINNING**

- **Health and safety course,** University of Sunderland 2019, (NHS) 2019
- **Medical writing course organised by Cyprus** 2019
- **Preparing to Teach (Career Development),** University of Sunderland 2017
- **Electro-hydrodynamic atomization course,** De Montfort University, Leicester 2017
- **Academic Writing course (2016),** University of Sunderland 2016